PHYSIOLOGICAL ASPECTS OF HYDRILLA [*Hydrilla verticillata* (L.f.) Royle] GROWTH AND REPRODUCTION

By

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In memory of George E. MacDonald; your love, support, guidance and understanding are the basis for all I have attained.

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PHYSIOLOGICAL ASPECTS OF HYDRILLA
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The goal of this research was to provide a better understanding of the fundamental processes involved in hydrilla growth and reproduction. Abscisic acid from hydrilla tissue was analyzed by enzyme-linked immunoassay. Although solid-phase extraction provided good purification of abscisic acid present in hydrilla tissue, no clean up of the extract was necessary prior to analysis. Crude, unpurified extracts of hydrilla stem and turion tissue were analyzed by ELISA. Validation of abscisic acid extraction and analysis procedure was verified by internal standardization. Accurate quantification could be obtained from plant extracts containing 0.02 to 5.0 pmol of abscisic acid. Short days promoted subterranean turion formation but this effect was reduced by long days and 5 and 10 ppb fluridone. Fluridone caused a significant reduction in chlorophyll and carotenoid levels and the abscisic acid

content of mature plant shoot tips. The concentration of abscisic acid in young plants was higher under short days. These studies provide further evidence that fluridone can be used as a fall herbicide treatment to reduce turion formation in hydrilla. The growth of both biotypes was reduced under short-day conditions. Monoecious hydrilla formed axillary and subterranean turions regardless of photoperiod, while dioecious hydrilla formed only subterranean turions only under short-day conditions. Fluridone reduced the growth of both biotypes and reduced turion production in monoecious hydrilla. Exogenously applied abscisic acid at 0.1, 1.0, and 10 μM induced axillary turion formation by dioecious hydrilla under long day conditions and abscisic acid at 1.0 and 10 μM induced axillary turion formation by monoecious hydrilla. This process could be reversed by gibberellic acid in both biotypes. However, subterranean turion formation was not observed under any treatment for the dioecious biotype, but was observed in control monoecious hydrilla plants. Monoecious hydrilla could be induced to flower by 50 μM gibberellic acid applications. This study indicates abscisic acid alone does not control axillary turion formation in monoecious and dioecious hydrilla, but rather a balance between abscisic acid and gibberellic acid may be the key to regulation of this phenological process.

CHAPTER I
INTRODUCTION

Hydrilla [Hydrilla verticillata (L.f.) Royle] is an exotic, submersed aquatic vascular plant that causes major problems in the freshwater ecosystems of Florida and the southeastern United States (Haller, 1976). Hydrilla interferes with navigation, flood control and most recreational water activities, including fishing, through a displacement of native vegetation (Langeland, 1990). Hydrilla was discovered in 1959 in Florida, in a Miami canal. It was also reported in Crystal River during the same period and was originally called Florida Elodea (Blackburn et al., 1969).

Hydrilla is a monotypic genus in the family Hydrocharitaceae, taxonomically an ancient monocot family, with the center of origin thought to be tropical Asia (Cooke and Luond, 1982). However, hydrilla is cosmopolitan, and is reported to be broadly distributed throughout Germany, Lithuania, England, Poland, the upper Nile of Africa, southeast Asia, Australia, Madagascar, India, China, Japan and the southern United States (Lazor, 1978). A review of hydrilla by Mitra (1955) provided the first detailed description of the species with historical background information. Linnaeus filius first described hydrilla as *Serpicula verticillata* and classified it in the Haloragaceae family. Hydrilla was later placed into the Hydrocharitaceae family under the name *Hydrilla ovalifolia* but it was later changed to *Hydrilla verticillata*.

Hydrilla has been characterized by many scientists, as indicated by several describing authors including Presl., Royle, and Casp. Today, *Hydrilla verticillata* (L.f.) Royle or Casp. is the scientific name used to denote hydrilla. Langeland et al. (1992) showed hydrilla to be endopolyploid, with monoecious hydrilla being triploid and dioecious hydrilla being diploid; but, evidence from root tip karyotypes suggest sexual compatibility across hydrilla populations and biotypes.

Anatomically, hydrilla is considered to be a primitive plant with reduced vasculature. The xylem tissue is vestigial and phloem members are greatly reduced (Yeo et al., 1984). The leaves are comprised of two contiguous epidermal layers with cuneate plastid inclusions, lacking in starch granules -- characteristic of most monocots. The upper epidermis is much thicker than the lower layer (Pendland, 1979). Hydrilla leaves are borne in whorls of 4 or 5, with a distinctive spiny midrib on the abaxial side of the leaf, distinguishing this species from elodea. Plants are attached to the hydrosoil through fibrous roots and may resprout from root crowns at the surface of the hydrosoil (Haller, 1976). Flowers are borne on stems near the water surface (Cooke and Luond, 1982). Male and female flowers may be borne on the same (monoecious) or separate (dioecious) plants. The control of floral production is not well known but Pieterse et al. (1984), reported that low nitrogen and phosphorus concentrations stimulated flower production in monoecious hydrilla.

Hydrilla regrows in the spring, as water temperature begins to increase, from root crowns and/or subterranean turions in the hydrosoil (Haller, 1976). Shoot growth is regulated by light quality with green light (prominent at deeper depths)

promoting stem elongation and red light (prominent at the surface) stimulating branching (Van et al., 1977). Hydrilla has also been shown to increase the level of chlorophyll b at greater depths, enabling the plant to use longer wavelengths of light for photosynthesis (Van et al., 1977).

The development of a thick, entangled growth at the water surface (mat) occurs when the shoots reach the water surface. This forms a dense canopy and reduces light penetration in the first 0.3 m by 95% (Haller and Sutton, 1975). The rapid growth of hydrilla has been reported to be 4.2 g dry $wt \cdot m^{2} \cdot day^{-1}$ (DeBusk et al. 1981). Hydrilla is extremely competitive, forming large, monotypic stands. Studies on the photosynthetic characteristics revealed hydrilla to have a CO_2 compensation point of 44 $\mu L \cdot L^{-1}$ with light saturation occurring at 600 to 700 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD (Van et al., 1976). Hydrilla can adapt to low light levels with a light compensation point of 10 to 12 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD (Bowes et al., 1977). Holaday and Bowes (1980) reported hydrilla could exist with low or normal CO_2 compensation points. Those plants with low compensation points had high activity of phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) and pyruvate, P_i dikinase (E.C. 2.7.9.1) enzyme levels, suggesting that hydrilla is capable of both C_3 and C_4 photosynthetic metabolism and the change was due to low CO_2 concentrations in the water (Barko and Smart, 1981; Holaday et al., 1983). Salvucci and Bowes (1983) implicated carbonic anhydrase (E.C. 4.2.1.1) in the reduction of photorespiration in hydrilla during periods of low CO_2 compensation points. Ascencio and Bowes (1983) also reported a major increase in PEP carboxylase under these conditions.

Collectively, these characteristics allow hydrilla to utilize the free CO_2 from the water sooner after sunrise than other aquatic plants due its low light compensation point. Furthermore, hydrilla can maintain net photosynthesis under low CO_2 levels through the activity of PEPcarboxylase. This increases hydrilla's competiveness, decreasing competition from other species and allows hydrilla to maintain the luxuriant growth of the mat under low CO_2 and high O_2 conditions. In addition, Kulshreshtha and Gopal (1983) reported allelopathic properties of hydrilla on species of *Ceratophyllum*, further increasing the competitive advantage of this species.

Several studies have evaluated the effect of mineral nutrition on hydrilla growth. Sutton (1985) suggested the growth of hydrilla is controlled by nutrients in the root zone, while Spencer (1986) showed hydrilla could grow equally well under low or high organic matter sediment composition. Stewart (1984) showed phosphorus in the rooting media was the limiting factor in growth for hydrilla. Basiouny and Garrard (1984) demonstrated that calcium and phosphorus uptake in hydrilla was passive while potassium, copper, iron and manganese uptake required metabolic energy. Iron appeared to be particularly important for hydrilla growth with tremendous iron uptake and an exceptionally high iron to manganese ratio required for optimum growth (Basiouny et al., 1977b). Basiouny et al. (1977a) showed iron was absorbed by roots and leaves of hydrilla and translocated throughout the plant.

Hydrilla is extremely difficult to manage and control because spread can occur through a variety of mechanisms including fragmentation and specialized dormant buds called turions (Sculthorpe, 1967). Langeland and Sutton (1980) reported

regrowth from hydrilla fragments with a single node and Sutton et al. (1980) reported one shoot tip will produce as much biomass as 16 tips. Seed production has been reported for the monoecious biotype but does not occur in dioecious hydrilla in the United States.

Turions can be formed in the axials of leaves, or at the ends of positively geotropic rhizomes which extend into the hydrosoil (Haller, 1976; Yeo et al., 1984). Subterranean turions (tubers) can remain dormant for as long as 5 years (Van and Stewart, 1990). Miller et al. (1976) reported that increased tuber size and density were correlated to increased water depth. There appears to be an environmentally enforced dormancy of tubers, preventing rapid depletion of the tuber bank (Van and Stewart, 1990). Basiouny et al. (1978b) showed gibberellic acid, ethephon, thiourea and storage at 5 C to increase turion sprouting, and hypothesized the existence of two types of dormancy in hydrilla subterranean turions. Stewart (1969) found gibberellic acid enhanced sprouting of subterranean turions while IAA and 2,4-D enhanced axillary turion development. Subterranean turions are thought to maintain hydrilla within a given area, possibly through periods of drought. Work by Basiouny et al. (1978a) showed that tubers are better able to survive drought than axillary turions with almost 17% germination rate for tubers dried for 64 hours.

Axillary turions are smaller than subterranean turions and are thought to function in dispersal (Thullen, 1990). These structures are generally formed on detached floating mats of hydrilla (Thullen, 1990; Miller et al., 1993). Furthermore, axillary turions could last only 1 year in the hydrosoil (Van and Stewart, 1990),

presumably due to their smaller size.

Turion formation in dioecious hydrilla has been shown to be a photoperiodic response with a critical photoperiod of 13 hours for subterranean turion formation (Van et al., 1978a). Haller et al. (1976) reported subterranean turion production occurred in north Florida between October and April. Klaine and Ward (1984) demonstrated the turion formation response was phytochrome mediated, with red light (650 nm) stimulation and far-red light (750 nm) repression of turion formation in the dioecious biotype. Exogenous ABA applications induced turion formation under non-inductive conditions. Van et al. (1978b) also showed exogenous ABA promoted turion formation under 16 hour daylengths. Axillary turion formation appears to respond similarly to subterranean turion formation with regard to photoperiod. Miller et al. (1993) showed hydrilla axillary turion production was similar to subterranean turion formation -- occurring in the fall and winter months only.

Both the monoecious and dioecious hydrilla biotypes occur in the United States. The dioecious female plant occurs throughout Florida, the southeast and California, whereas monoecious hydrilla is found in Washington D.C., Virginia, Maryland and North Carolina. Besides a difference in flowering, there seems to be several distinct differences between the two biotypes in terms of vegetative growth and turion production.

Monoecious hydrilla produces subterranean turions under both 10 and 16 hour photoperiods, with a greater number of turions produced under short-day conditions.

The growth of monoecious hydrilla is generally prostrate, near the hydrosoil with many horizontal stems and higher shoot densities than dioecious hydrilla. In comparative studies, Steward and Van (1987) found that the monoecious biotype tolerated cooler temperatures and was able to sprout at lower temperatures. Ames et al. (1986) also reported greater growth of monoecious hydrilla under cooler temperatures compared to dioecious.

Subterranean turion production has been shown to be greatest under short days for monoecious hydrilla with more turions produced than the dioecious form, but there was a decline in biomass accumulation with short days. Monoecious hydrilla also appears to able to reproduce much more rapidly than dioecious, forming new subterranean turions in 4 weeks as compared to 8 weeks for dioecious hydrilla (Van, 1989). Spencer et al. (1987) reported subterranean turions to be nearly 2 to 5 times as large as axillary turions, with monoecious subterranean turions smaller than those of dioecious hydrilla. In addition, Ames et al. (1987) showed percent sprouting was higher in monoecious than dioecious hydrilla. These studies indicate monoecious hydrilla possesses an annual growth habit, along with rapid and prolific turion production, adapting this biotype to northern areas which have cooler temperatures and short growing seasons.

Earlier work suggested monoecious hydrilla was photoperiodic in response to turion formation. Spencer and Anderson (1987) showed that monoecious hydrilla did not produce subterranean turions under daylengths greater than 14 hours and production increased as daylength decreased. They also reported photo-interruption

of monoecious hydrilla inhibited turion formation, similar to long-day grown plants (Anderson and Spencer, 1986). However, monoecious hydrilla appears only to be delayed in the formation of turion under long-day conditions, producing turions after 8 weeks under long-day conditions (Van, 1989).

Management of hydrilla is difficult due to rapid growth rate and prolific turion formation. Cultural management schemes, such as drawdowns to deplete tuber populations have had limited success (Haller et al., 1976) and biocontrol agents, such as grass carp, are unpredictable forms of control. Martyn (1985) used grass carp to control 9000 acres of hydrilla in a 20,000 acre lake in Texas. All hydrilla was removed after 2 years but over 270,000 fish were necessary. Insect biocontrol has been studied, with several introductions but these have met with only limited success.

The herbicides diquat, endothall and copper sulfate, alone or in combination provide good initial control but regrowth of the hydrilla quickly occurs. Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) has been shown to be very effective for hydrilla control, but contact time and concentration are critical issues affecting the efficacy of a given fluridone treatment (Van and Stewart, 1985; Haller et al., 1990; Fox et al., 1991).

Fluridone affects plant tissue by decreasing pigment levels (Maas and Dunlap, 1989). This is due to a decrease in carotenoid levels followed by a subsequent decrease in chlorophyll content due to excess photooxidative stress (Devlin et al., 1978). Bartels and Watson (1978) demonstrated the mechanism of action of fluridone to be carotenoid inhibition and this effect could be overcome by adding δ-

aminolevulinic acid (Fletcher et al., 1984). The actual site is an inhibition of the conversion of phytoene to phytofluene in the terpenoid biosynthetic pathway (Mayer et al., 1989). Fluridone was reported to be a non-competitive enzymatic inhibitor, with reversible binding. Fluridone causes similar effects on hydrilla tissue, decreasing the chlorophyll and carotenoid content, but interestingly, increasing anthocyanin content (Doong et al., 1993).

Fluridone was originally marketed for weed control in cotton (Gossypium hirsutium L.). Translocation studies showed fluridone to be translocated apoplastically following root absorption. Tolerant species, such as cotton, show limited translocation and root uptake (Berard et al., 1978). Banks and Merkle (1978) reported good control with fluridone of many annual weeds in cotton but also reported some soil persistence.

Fluridone is an off-white crystalline solid, with a melting point of 151-154 C and a water solubility of 12 ppm (McCowen et al., 1979). It is classified as a pyridinone herbicide with a molecular weight of 329.3 (McCowen et al., 1979). Mossler et al. (1991) reported microbial breakdown for fluridone was very slow and variable. Aqueous degradation rates from lake collected microbial consortia did not correlate to previous fluridone use patterns in the aquatic environment. Fluridone has a fairly high affinity to clay or organic matter (Mossler et al., 1993). Therefore, slow-release formulations could only be effectively used in aquatic ecosystems with a sandy bottom substrate or in situations where the fluridone pellet does not contact the hydrosoil.

Ultraviolet light breakdown is the major degradation mechanism in the natural environment. Photolytic degradation of fluridone is highest at 297 to 325 nm with a half-life of 26 hours although some degradation can occur at 325 to 355 where the photolytic half-life was 840 hours (Mossler et al., 1989).

Fluridone has also been shown to decrease the levels of ABA within plant tissue (Jones and Davies, 1991). Abscisic acid is derived from carotenoid precursors so the negative effect of fluridone on carotenoid levels also translates to lower ABA content. Abscisic acid is thought to be involved in turion formation in hydrilla, and fluridone could be used to regulate this process. Turion formation in hydrilla can be inhibited by fluridone under short-day conditions at concentrations greater than 5 ppb (MacDonald et al., 1993), but the effect on ABA and subsequent inhibition of turion formation was not determined.

Abscisic acid (ABA) is a plant hormone involved in a multitude of physiological responses including stomatal opening (Cornish and Zeevaart, 1985), bud and seed dormancy (Quatrano, 1987; Barros and Neill, 1986), freeze tolerance (Johnson-Flanagan et al., 1991; Reaney and Gusta, 1987), and tuber and turion formation (Smart and Trewavas, 1984). ABA is formed through the carotenoid biosynthetic pathway by the conversion of 9'-*cis*-neoxanthin to xanthin (Xan). Xanthin is subsequently converted to ABA aldehyde and finally to *cis*-ABA (Gamble and Mullet, 1986; Zeevaart et al., 1989; Li and Walton, 1990; Parry and Horgan, 1991). Light can cause isomerization to *trans*-ABA but only *cis*-ABA is thought to be the biologically active form. ABA biosynthesis is regulated by the production of

xanthin, not the conversion of xanthin to ABA (Parry and Horgan, 1991).

ABA has been implicated in the seed dormancy of many species (Hole et al., 1989; Singh and Browning, 1991; Le Page-Degivry and Garello, 1992), but the exact mechanism not well understood. Morris et al. (1991) indicated that ABA induced dormancy in wheat seeds at the gene level while Ozga and Dennis (1991) showed changes in ABA in apple seeds were not related to dormancy. In a review, Quatrano (1987) reported that ABA has been shown to modulate gene expression in cultured embryos of certain crops. During seed development the increase in endogenous ABA promotes maturation of the embryo and represses seedling growth. This is accomplished by modulating gene sets, positively affecting the maturation set and negatively affecting the germination set. As the seed begins to lose water, the amount of ABA and the embryo sensitivity to ABA decreases and the dehydration of the seed, not ABA, prevents germination.

Subbaiah and Powell (1992) reported that the chilling requirement of apple seed is not related to ABA changes during stratification, and chilling, not ABA, was required for germination. Also the decline in ABA was largely due to leaching of the seed coat and nuclear membrane while ABA content of embryo remained constant. They postulated that ABA leaching from the seed allows certain promotive forces to develop, but these can only occur after the chilling requirement was met.

Earlier work also supports this contention. Schopfer et al. (1979) showed ABA could reversibly arrest embryo development by inhibiting water uptake, not by control of DNA, RNA or protein synthesis. Release from dormancy is not due to

lower ABA levels, but rather to a loss of sensitivity to ABA. Oishi and Bewley (1990) found the decline in ABA will permit germination of maize kernels, but only drying will allow the aleurone layer to become sensitive to gibberellic acid. Gibberellic acid induces α-amylase activity, causing the remobilization of food reserves for seedling growth.

Mutations are often used to study the role of ABA. Koornnef et al., 1989 used three ABA mutants of *Arabidopsis thaliana*, termed *abi* loci, and found all mutations reduced dormancy. However, one mutation did not effect the water relations of leaf tissue. They hypothesized that the mutations affect different tissue-specific receptors. Research by McCarty (unpublished) indicated that there was an ABA receptor molecule complex on a promoter sequence of RNA. When ABA binds to the promotor, RNA transcription begins and a specific enzyme(s) or event is initiated.

There have been several indications that ABA plays an important role in plant growth, especially under water-stressed conditions. Saab et al. (1990) showed that ABA maintains primary root growth and inhibits shoot growth of maize seedlings under low water potentials. In later work, Saab et al. (1992) suggested a differential response to endogenous ABA by root and mesocotyl growing zones of maize. At low water potentials, ABA had the greatest effects near the root tip and this effect decreased from the tip. Mesocotyl tissue is more responsive to ABA in those cells away from the meristematic region. Porter (1981) suggested that endogenous levels of ABA in different plant organs could be a factor in the directional control of

assimilate transport in plants. Hoffmann-Benning and Kende (1992) reported that the growth of rice was regulated by a ratio of gibberellic acid and abscisic acid, as a growth promoter and inhibitor, respectively.

Interesting work on the effects of ABA on aquatic macrophytes has also been reported. Kane and Albert (1987) demonstrated that ABA, possibly as an indicator of water-stress, caused changes in aerial leaf morphology and vasculature in *Hippuris vulgaris*. ABA also caused aerial leaf development in *Myriophyllum* and *Proserpinaca*, and suggested possible ABA/ethylene interactions (Kane and Albert, 1989). Goliber and Feldman (1989) measured the ABA content of the aerial leaves of *Hippuris vulgaris* and showed much higher levels. They suggested the effect of osmotic stress is the cue in aerial leaf development.

There is considerable changes in the compartmentation of ABA during light and dark periods due to pH changes, with a 2-fold increase of ABA in the apoplast in the dark. ABA can irreversibly conjugate to glucose forming ABA-glucoside. This form is inactive and is restricted to the vacuole, and may be a sequestration mechanism by which ABA is removed from the active pool. ABA has also been shown to be a major factor in the control of stomatal opening through regulation of guard cell turgor pressure (Zeevaart and Creelman, 1988). Guard cells under stress appear to release ABA, initiating closure. Water-stressed roots can also produce ABA that is translocated to the leaves and effects stomatal closure (Zeevaart and Creelman, 1988).

Hartung (1983) reported the site of action of ABA in guard cells to be on the outer surface of the plasmalemma. Raschke (1987) reported ABA prevented stomatal opening by blocking H^+ extrusion and K^+ influx, and initiated rapid closure by K^+ release. Recent work suggested that ABA acts on a G-protein at the membrane surface, activating phospholipase C, which catalyzes the hydrolysis of phosphotidalinositol-pyrophosate to inositol-3-phosphate (IP_3) and diglyceralhyde. IP_3 then causes intracellular Ca^{2+} release, blocking K^+ channels and lowering stomatal opening. There is also some evidence that internal ABA may have some control over this process as well.

Abscisic acid is also thought to be involved in the process of turion and tuber formation in hydrilla and other species. Tubers are among the most common form of vegetative reproductive structures and are produced by several species including potato (Solanum tuberosum) and jerusalem artichoke (Helianthus tuberosum). Tubers are swollen underground stems possessing multiple nodes and greatly reduced leaf characteristics (Cutter, 1978). In potato, the morphology of tuber formation has been well documented. Tubers are formed from stem tissue, generally at the ends of basal stolons (Peterson, et al., 1985), and are initiated in the youngest elongating internode (Peterson, et al., 1985). The pith cells in this area enlarge, followed by rapid cell division of the perimedullary parenchyma and cortical cells. Cell division occurs in early tuber development and final tuber size is achieved through cell enlargement (Reeve et al., 1973).

Turions are similar to tubers but are morphologically less complex, derived from leaf tissue, with little differentiation of the stem tissue. Turion are often found in freshwater aquatic species, such as green milfoil (Myriophyllum verticillatum L.) (Weber and Nooden, 1976) and giant duckweed (Spirodella polyrhiza) (Smart and Trewavas, 1983). Unlike tubers, turions sprout from a single node at the tip of the structure, although turions are comprised of several densely packed nodes (Yeo et al., 1984). The outer portion of the turion is made up of leaf tissue, which swells and envelopes the terminal apex, giving the structure a scale-like appearance. These leaves accumulate carbohydrates, with an abscission layer forming at the base of the lowermost leaves. The turion detaches from the mother plant and remains dormant until conditions favorable for growth arise.

Turion formation by dioecious hydrilla is promoted by short-days (Haller et al., 1976). This response has been shown to be phytochrome mediated (Klaine and Ward, 1984). Phytochrome has been implicated in several plant processes, including flowering, seed germination and tuber formation (Quail, 1991). However, recent work provided evidence that phytochrome may regulate more fundamental plant processes (Lissemore et al., 1987), (Smith and Whitelam, 1990), (Kendrick and Nagatani, 1991), (Seeley et al., 1992) and (Edgerton and Jones, 1992). Phytochrome appears to be involved in the activation of K^+ channels (Lew et al., 1992) and has been suggested to be a protein kinase (McMichael and Lagarias, 1990) (Doshi et al., 1992). Recent research also indicated that at least two types of phytochrome [Type I (Phytochrome A) or Type II (Phytochrome B] can be present within plant tissues

(Abe et al., 1985) (Shimazaki and Pratt, 1985). Phytochrome A has been shown to regulate stem elongation (Boylan and Quail, 1991), chloroplastic gene expression (Sharrock et al., 1988) and anthocyanin biosynthesis (Adamse et al., 1988). Phytochrome B has been linked to photoperiodic responses (Vince-Prue and Takimoto, 1987; Takimoto and Saji, 1984) with respect to floral induction.

Changes in hormone levels occur soon after photoperiodic induction of tuber formation. In potato, changes in ABA, gibberellin, and cytokinin levels have been reported. Gibberellins are high under long-day photoperiodic conditions and decline under short days (Krauss, 1985), while the opposite effect is observed with abscisic acid. Several researchers have reversed the effect of photoperiod with exogenous applications of these hormones (i.e. preventing tuber formation under short-day lengths with gibberellic acid while inducing tuberization with abscisic acid under long-day lengths). However, a critical ratio or balance between these two hormones may be needed for tuberization to occur (Menzel, 1980) (Vreugdenhill and Struik, 1989). Cytokinins accumulate in response to tuberization but do not induce this process (Krauss, 1985). In addition, increased nitrogen levels in the leaves have been shown to delay or inhibit tuber formation in potato (Krauss, 1978), while increased photosynthate concentration promotes this response (Wenzler et al., 1989).

Exogenously applied abscisic acid also induced turion formation in hydrilla (Van et al., 1978), (Klaine and Ward, 1984). Klaine and Ward (1984) also reported that turion formation in hydrilla was reduced by exogenous gibberellic acid, similar to that observed for potato. However, cytokinin had no effect. Turion formation in

giant duckweed (Perry and Byrne, 1969) can also be induced by exogenous ABA applications, suggesting that the role of abscisic acid in regulating vegetative reproduction is similar for a variety of species.

The role of ABA and gibberellic acid in tuber formation is well documented but specific changes related to initiation of this process is unclear. Hannapel et al. (1985) showed exogenous gibberellin prevented the accumulation of patatin and two other potato tuber proteins and correlated this with the inhibition of tuber formation. Abscisic acid had an inhibitory effect on overall protein synthesis in Spirodela. polyrhiza turion formation (Smart and Trewavas, 1984), and the authors suggested that there were several novel proteins specific to induced tissue.

Quantification of abscisic acid has traditionally been accomplished through the use of gas chromatography-mass spectrometry (Dumbroff et al., 1983; Reymond et al., 1987). However, the use of enzyme-linked immunoassays (ELISA) for ABA analysis has increased over the years. The advantage of ELISA is that limited sample purification and increased number of samples can be analyzed within a given time frame.

Several researchers have developed ABA antibodies (Weiler, 1980; Mertens et al., 1983; Ross et al., 1987; Quarrie et al., 1988). Most monoclonal antibody immunoassays were able to use crude aqueous extracts without interference, providing accurate quantification comparable to GC-MS analysis (Leroux et al., 1985; Quarrie et al., 1988; Soejima et al., 1990; Tahara et al., 1991). Some radioimmunoassays can detect free and conjugated ABA; this is dependant on C_1

or C_4 ABA coupling to proteins for total or free ABA, respectively (Weiler, 1980).

However, ABA analysis with ELISA from plant tissues varies with plant species and tissue type. Some extracts may require purification while other extracts do not. Therefore, validation through internal standardization is recommended for ABA analysis from a previously unvalidated tissue type.

Hydrilla is a perennial species, with regrowth from overwintering turions often being the main source for re-establishment. Turions sprout in the spring, forming a large, monotypic stand. The hydrilla then reproduces in the fall, replenishing the turion supply within the hydrosoil. Therefore, turion depletion in the hydrosoil would be the most effective means for long-term hydrilla control. However, herbicide treatments during fall conditions (turion formation) would result in applications to established hydrilla plants. However, fluridone has been shown to be very effective for the control of hydrilla, and can reduce turion formation at concentrations greater than 5 ppb. Fluridone will cause decreased ABA levels within plant tissues and this may be the reason for the reduction in turion production.

The ability of hydrilla to reproduce by turions is the greatest restraint to the control of this species. Therefore, a better understanding of the fundamental processes involved in turion initiation and development would greatly assist in efforts to effectively manage this species. To accomplish this goal the objectives of this research were to 1) develop an ELISA based ABA analysis procedure for quantifying ABA from hydrilla; 2) test the hypothesis that fluridone reduces turion formation from a reduction in ABA content, not a lethal impact on plant growth; and 3)

compare the response (growth and turion development) of monoecious and dioecious hydrilla biotypes cultured under controlled conditions to long and short-day conditions, fluridone and exogenous plant growth regulators.

CHAPTER II
EVALUATION OF AN ENZYME LINKED IMMUNOASSAY PROCEDURE FOR THE ANALYSIS OF ABSCISIC ACID IN HYDRILLA

Introduction

Abscisic acid (ABA) has been implicated in many plant developmental processes, including control of gene expression, plasma membrane ion flux, and seed dormancy (Neill et al, 1986; Barratt et al., 1989). In addition, ABA plays a vital role in plant water relations, through the regulation of stomata and growth under water stressed conditions (Saab et al., 1990; Saab et al., 1992). ABA has also been implicated in turion formation and heterophylly of aquatic macrophytes (Smart and Trewavas, 1984; Kane and Albert, 1987).

Abscisic acid is a 15-carbon compound with a molecular weight of 264.3. It exists in two forms: 'cis' and 'trans', which isomerize in light. Cis-ABA is the biologically active form, therefore quantification of ABA should take this fact into account. The acidic nature of abscisic acid (pK_a of 4.8) has been utilized in analysis protocols (Jones and Davies, 1991).

Abscisic acid is derived from the terpenoid biosynthetic pathway, but the exact synthesis route has only recently been elucidated. Earlier work suggested ABA was formed through two separate pathways: a direct and indirect pathway (Walton, 1980). The direct pathway was suspected to involve a C_{15} precursor derived from farnesyl-pyrophosphate, which may itself be xanthoxin (Milborrow, 1983). Xanthoxin

was discovered in the late 1960's (Taylor and Burden, 1973) as a precursor to ABA and led to the indirect pathway hypothesis, as this compound is derived from the breakdown of carotenoids. Therefore, ABA was also proposed to be derived from carotenoid breakdown. However, elucidation of the exact biosynthetic pathway has been extremely difficult due to the large number of potential ABA precursors versus the minute amount of ABA (Cowan and Railton, 1986; Milborrow, 1983).

Subsequent research has provided the most definitive evidence that ABA is produced via the indirect pathway of carotenoid breakdown. Biosynthetic inhibitors, such as fluridone, have been shown to block carotenoid synthesis and also lower ABA levels (Moore and Smith, 1984; Gamble and Mullet, 1986; Oishi and Bewley, 1990). In addition, carotenoid deficient mutants have lower levels of ABA (Wang et al., 1984; Neill et al., 1986). Since these studies, most researchers have concluded the synthesis of ABA involves epoxidation and isomerization of specific carotenoid molecules, the formation of xanthoxin, and subsequent ABA formation (Zeevaart et al., 1989; Li and Walton, 1990; Parry and Horgan, 1991).

Abscisic acid can be extracted from plant tissue through a variety of methods. Goliber and Feldman (1989) used 80% methanol for extraction from Hippuris vulgaris while Gamble and Mullet (1986) found adequate extraction with 100% acetone. Loveys and vanDijk (1988) found boiling water sufficient for extracting ABA from grape (Vitis spp.) leaves and Tahara et al. (1991) found extraction with tris-buffered saline to provide excellent extraction of ABA from wheat (Triticum aestivum L. em. Thell.) leaves.

Traditional methods of ABA quantification include high performance liquid chromatography (HPLC) and/or gas-liquid chromatography (GC). These methods provide excellent quantification but require a large amount of purification (Dumbroff et al., 1983; Leroux et al., 1985; Soejima et al., 1990). Recently, enzyme-linked immunoassays (ELISA) have been developed for ABA detection, greatly reducing the rigorous purification required prior to analysis (Raikhel et al., 1987; Tahara et al., 1991). In addition, the number of samples that can be processed at a single time is much greater with ELISA.

Hydrilla [*Hydrilla verticillata* (L.f.) Royle] is a submersed aquatic plant that has become a weed problem throughout much of southeastern United States and California (Langeland, 1990). Hydrilla is a highly aggressive species, forming large, monotypic stands that interfere with flood control, navigation and many recreational water activities including boating and fishing (Haller, 1976; Langeland, 1990). Hydrilla persists within a given area primarily through the production of specialized vegetative reproductive structures called turions. Turion formation in hydrilla and several other aquatic species is thought to be directly affected by ABA but the exact role of ABA is not known (Van et al., 1978; Klaine and Ward, 1984). Therefore, a better understanding of the affect of ABA on turion formation in hydrilla could provide more effective management strategies for this noxious species.

Several researchers have found the enzyme-linked immunoassay (ELISA) procedure specific enough to quantify ABA in crude extracts without purification (Tahara et al., 1991). However, each individual plant species must be tested and

validated if crude extraction is to be used (Zeevaart and Creelman, 1988). In addition, plant tissue types may also vary in the content of interfering compounds, particularly between chlorophyllous and achlorophyllous tissue. Therefore, the major impediment to the universal applicability of ELISA is the unpredictable presence of compounds that cross-react with the ABA-specific antibody. A procedure known as internal standardization must be conducted on tissue prior to analysis for ABA using ELISA. This technique allows a determination of whether purification of the sample is necessary before ELISA quantification.

Although ABA has been implemented in hydrilla turion development, ABA quantification from hydrilla tissue by ELISA has not been widely used. Therefore, studies were conducted to develop a purification procedure for detecting ABA from hydrilla and to determine if such a procedure was warranted for quantification by ELISA.

Materials and Methods

Elution profiles and column/ABA characterization. Reverse-phase cartridges, particularly C_{18}, have been widely used for abscisic acid purification (Hubick and Reid, 1980). Therefore, initial studies were performed to characterize the relationship between eluant volume, methanol:water ratio and pH on the retention of ABA to this type of column. Columns[1] were prepared prior to use by washing

[1] PrepSep; Fisher Scientific, Pittsburgh, PA 15219.

once with methanol and twice with deionized water. All samples were passed through the column under vacuum (90 kPa) and the resulting eluant was collected in 20 ml glass vials. During elution these vials were placed within a vacuum manifold[2].

In the first experiment, 100 μL of 1 mM ABA was added to the top of the column and eluted with varying concentrations of methanol:water (20:80, 50:50, 70:30, 80:20, 100:0) in 10 mL aliquots up to 50 mL. The elution pH was approximately 7, with the majority of the ABA molecules in the protonated form. The ABA content was then determined spectrophotometrically at 250 nm. Recovery was expressed as a percent of ABA added.

A similar experiment was conducted with varying concentrations of methanol:water (0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 70:30, 80:20, and 100:0) in 10 ml aliquots up to 50 mL. In addition, the methanol:water elutions were acidified with 1% formic acid to obtain a pH of less than 3.0. This resulted in most the ABA in an unprotonated, neutral form. Recovery was expressed as a percent of ABA added.

The preceding elution studies allowed the development of a purification and concentrating procedure that was used in the following two experiments (see results and discussion). An experiment was conducted to determine if the presence of compound(s) in hydrilla extract would interfere with accurate quantification of ABA

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[2] PrepTorr; Fisher Scientific, Pittsburgh, PA 15219.

using UV spectroscopy. Tissue extract contained 20 mg-fresh weight hydrilla shoot tips which were homogenized in 10 to 15 mL 100% methanol and the extract filtered under vacuum. This extract contained three levels of plant tissue (0, 12.5, and 25 mg-fresh weight/sample). Abscisic acid was added to the plant extracts at concentrations of 0, 8, 13, or 18 nM prior to column extraction. ABA was detected spectrophotometrically at 250 nm.

A study was also conducted to determine if components in hydrilla extract would influence the recovery of ABA purified using column chromatography. This study involved fortifying hydrilla extract with different concentrations of ABA before or after purification. Tissue extract was prepared using the aforementioned methods and three concentrations (150, 250, or 350 nM) of ABA were used for fortification. Abscisic acid recovered was detected spectrophotometrically at 250 nm.

ELISA internal standardization for ABA in hydrilla. An internal standardization experiment was conducted to determine if purification of hydrilla tissue was necessary prior to ABA quantification with ELISA. Antibody and antibody tracer specific for ABA were obtained directly from the manufacturer[3]. Procedures for the preparation of ELISA plates, sample preparation and ABA concentration determination are outlined in Appendices A-E.

Internal standardization was performed on crude extracts of hydrilla stem and turion tissue utilizing ELISA. Due to the large range of values to be tested,

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[3] Phytodetek™, Idetek, Inc., 1245 Reamwood Ave., Sunnyvale, CA 94089.

standardization of each tissue type was performed in two experiments. In the first experiment, 100 μL of 0, 0.02, 0.04, 0.06, 0.08, 0.2, 0.4, or 0.6 pmol ABA was added to dilutions of hydrilla stem tissue (1:2 and 1:4) and turion tissue (1:4 and 1:8). Dilutions were based on 20 mg-dry weight/sample as a 1:1 dilution and ABA was added prior to quantification by ELISA. Samples were extracted according to the procedures outlined in the previous section. The methanolic extract was then dried under a stream of nitrogen gas and reconstituted in tris-buffered saline prior to quantification by ELISA.

In the second experiment, 100 μL of 0.0, 0.2, 0.5, 1.0, 2.0, or 5.0 pmol ABA was added to dilutions of hydrilla stem tissue (1:1 and 1:3) and turion tissue (1:1 and 1:4). Dilutions were based on 12 or 8 mg-dry weight/sample as the 1:1 dilution) for stem or turion tissue, respectively. ABA was added prior to quantification by ELISA. Tissue extracts for both experiments were prepared in a manner similar to plant material used in the column extraction procedure.

Results and Discussion

Abscisic acid under neutral pH conditions showed little affinity for the C_{18} column when the mobile phase contained greater than 30% methanol. The majority of ABA was recovered with only 10 mL of 30% methanol (Table 2.1). Much higher concentrations of methanol were necessary to elute ABA from the C_{18} column when the mobile phase was acidic (Table 2.2). Under low pH conditions ABA retention increased at similar methanol concentrations to the previous experiment. However, complete retention was observed at concentrations below 20% methanol.

Table 2.1. The effect of elution volume and methanol concentration on abscisic acid C_{18} column retention under neutral pH conditions.

Cumulative Elution Volume	Methanol Concentration (%)								
	0	10	20	30	40	50	70	80	100
mL	% ABA eluted								
10	8±3[1]	50±4	77±2	87±2	94±1	94±3	89±5	76±3	80±4
20	33±3	27±2	18±2	2±2	0	0	--	5±3	5±3
30	15±2	11±1	2±1	2±2	0	0	0	0	0
40	7±1	6±2	2±1	0	0	0	0	0	0
50	4±1	0	0	0	0	0	0	0	0

[1] Means (four replications) followed by standard errors.

Table 2.2. The effect of elution volume and methanol concentration on abscisic acid C_{18} column retention under low pH conditions.

Cumulative Elution Volume	Methanol Concentration (%)								
	0	10	20	30	40	50	70	80	100
mL	% ABA eluted								
10	0	0	0	2±1[1]	14±6	57±2	90±3	99±2	92±3
20	0	0	0	0	59±3	44±3	15±3	--	11±4
30	0	0	0	8±4	13±4	0	0	0	0
40	0	0	0	28±4	0	0	0	0	0
50	0	0	0	22±3	0	0	0	0	0

[1] Means (four replications) followed by standard errors.

Concentrations greater than 50% removed ABA from the column after 20 mL of elution volume. Extraction of ABA using C_{18} solid-phase columns is highly dependent on pH. Low pH conditions allows greater retention of the ABA to the column while neutral conditions do not. These results agree with the work of Parry and Horgan, 1991 and other researchers.

The purpose of these studies was to develop a two-stage procedure for the purification and concentration of ABA for analysis by ELISA. In the first purification step, it was imperative that the appropriate solvent strength (% methanol) be achieved to allow the retention on the column of as many extract components as possible while allowing the elution of ABA. Because the volume of solvent increased in this step, it was then necessary to concentrate the purified ABA extract. This was accomplished by determining the appropriate solvent strength of the mobile phase that would allow the retention of ABA by the column. This technique is known as solid phase extraction and has many advantages over liquid to liquid extraction, which results in large quantities of waste solvent. In addition, solid phase extraction is cheap, rapid and allows for the simultaneous preparation of numerous samples. The results provided information necessary to devise a clean-up procedure that could be utilized for ABA analysis. This procedure provided over 92% recovery for all concentrations when ABA was extracted in the absence of plant tissue (data not shown). Details of this procedure can be found in Appendix F.

When this procedure was used with the addition of plant tissue, it resulted in greater than 89% recovery for all concentrations of ABA added (Table 2.3). Visual

Table 2.3. The effect of plant tissue and column extraction on the recovery of abscisic acid. Abscisic acid was added before or after column purification.

ABA Added	Plant + ABA Before Column	Plant + ABA After Column	Recovery
(nM)	---------- absorbance ----------		----%----
0	0.276±0.40[1]	0.276±0.40	--
150	0.580±0.20	0.632±0.34	92
200	0.703±0.09	0.752±0.45	93
250	0.786±0.18	0.880±0.42	89

[1] Means (three replications) followed by standard errors.

observations indicated that the column purification procedure provided good separation of chlorophyll and carotenoids. However, anthocyanins were extracted with the ABA, as indicated by a pinkish color during the second column extraction.

Furthermore, the presence of interfering compounds in hydrilla tissue extract was not detected using this procedure (Figure 2.1). An internal standardization procedure allows for the detection of an interfering compound. If the addition of ABA to various extract dilutions does not cause a change in the rate of absorbance then interfering components are not present. The absence of such a compound would be indicated by parallel lines as the rate at which ABA detected as a function of ABA added was the same regardless of the extract dilution. Although different amounts of ABA were detected, dilutions as a function of ABA added was the same.

The column clean-up procedure developed reported herein provided good recovery of ABA and separation of possible interfering compounds. Although additional purification could be necessary with chromatographic methods of quantification, this procedure provided an excellent initial purification step. Furthermore, this procedure provided adequate purification of hydrilla tissue for quantification by ELISA, if purification was needed.

Internal standardization of ELISA allowed for the detection of an interfering compound (s) by dilution of the interfering compound (s) with the addition of higher levels of ABA. The absence of interference would be indicated by parallel lines of the two levels of plant tissue and/or the lack of a significant interaction ($P > 0.05$). The results from hydrilla stem tissue indicated the absence of interfering compounds

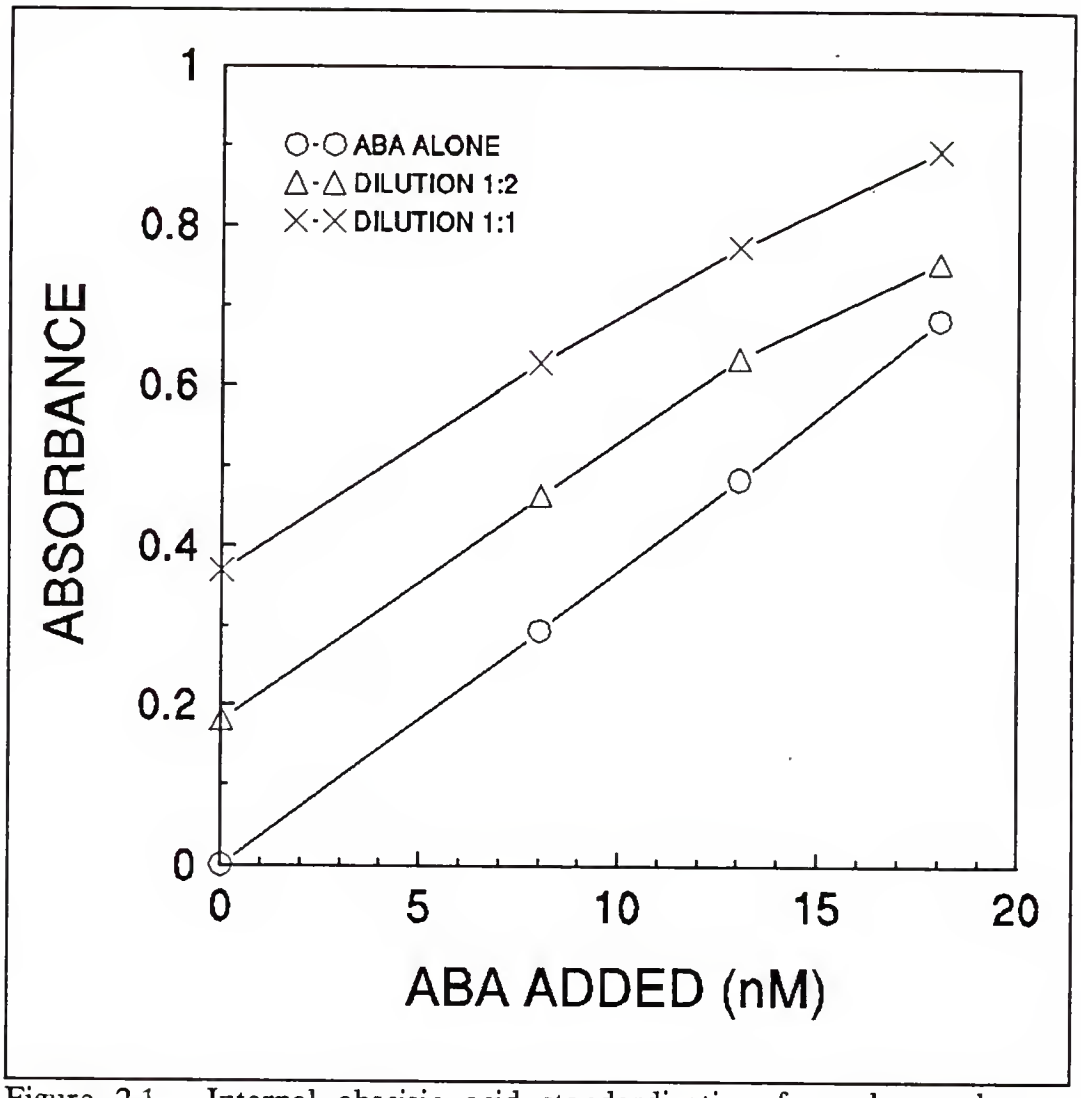

Figure 2.1. Internal abscisic acid standardization for column cleanup procedure with hydrilla tissue.

(Figures 2.2 and 2.3). Good separation of the two dilutions was observed in the first experiment at 0.6 and 0.8 pM ABA. However, the ability to detect differences in dilution was limited to a range of 0.2 to 0.5 pM in the second experiment. Hydrilla turion tissue also appeared to be free of interfering compounds (Figures 2.4 and 2.5). Although the lines do not appear to be absolutely parallel, statistically there was no interaction ($P > 0.05$) between ABA added and ABA detected for the different extract dilutions. This implies that there was a consistent change in the amount ABA detected regardless of the amount of ABA added (i.e. the lines representing independent and dependent variables for the different dilutions were parallel although differing in magnitude).

Furthermore, the presence of interfering compounds would result in response convergence at a higher added ABA, because the interfering compound (s) would react with the antibody, over-estimating ABA present. However, these data do not fit this scenario. In addition, preliminary experiments were conducted by adding ABA prior to purification and the same relationship between ABA added and ABA detected was observed (data not shown). Based on these results analysis of ABA can be accurately determined from hydrilla tissue by analysis of crude extraction. However, prudence should be exercised during analysis to ensure proper dilution of the respective sample; only those readings within the linear range of the standard curve are valid. The internal standardization reported herein provided an accurate determination within the range of 0.02 to 5.0 pmol per sample, allowing a broad range of sample ABA concentrations to exist. This also provides a large degree of

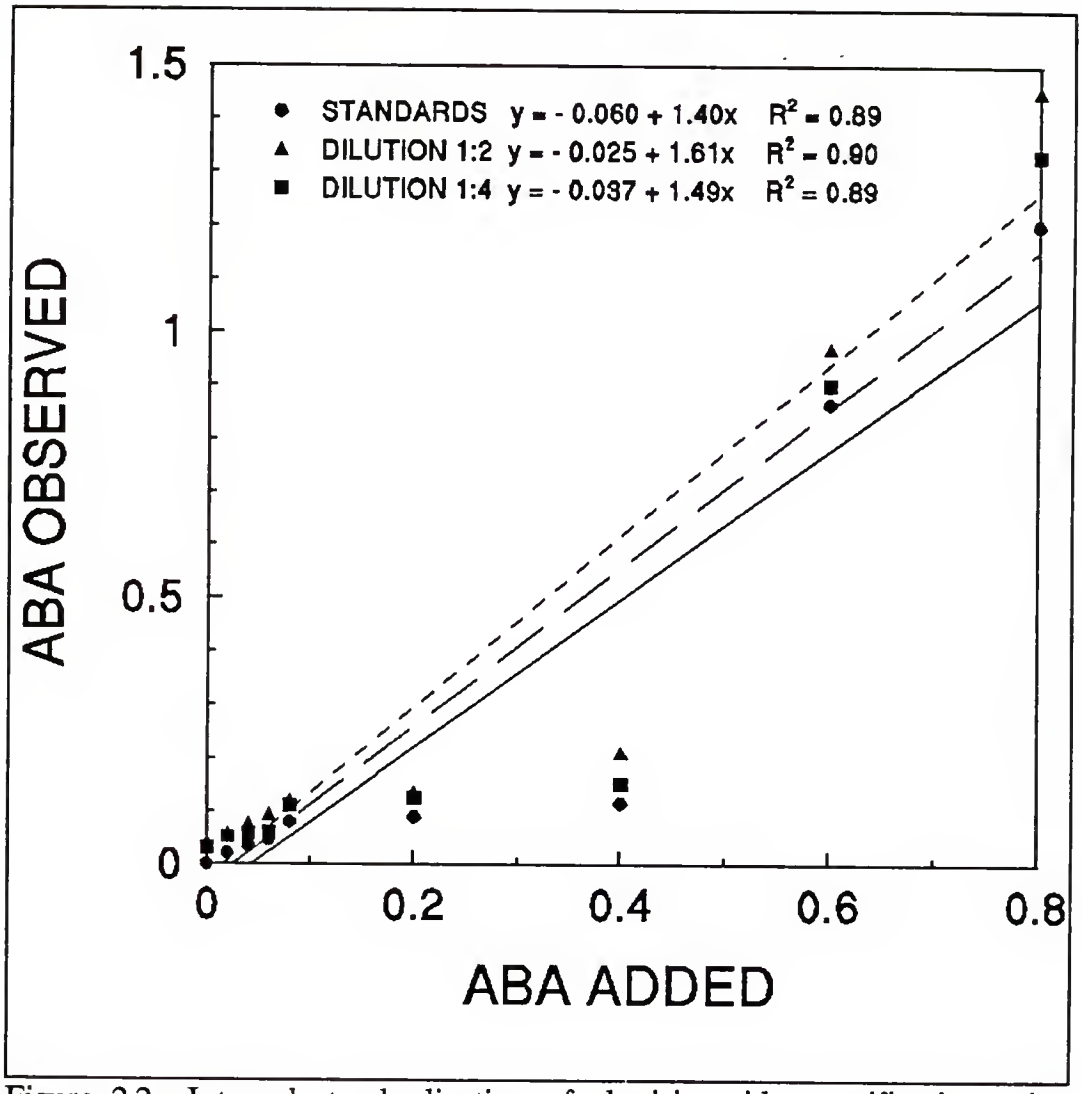

Figure 2.2 Internal standardization of abscisic acid quantification using ELISA with hydrilla stem tissue. Range of values was 0.02 to 0.8 pmol.

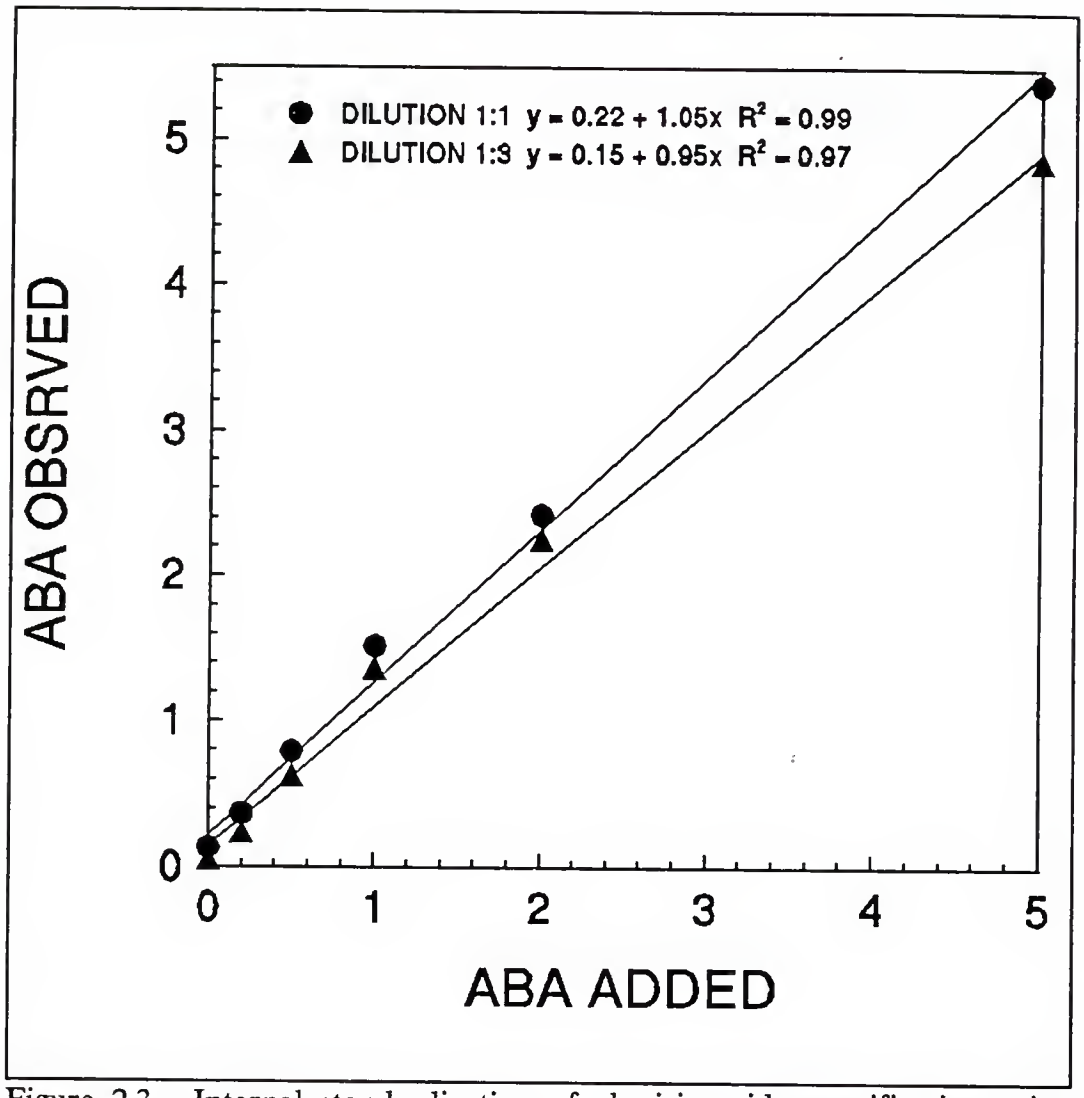

Figure 2.3. Internal standardization of abscisic acid quantification using ELISA with hydrilla stem tissue. Range of values was 0.2 to 5.0 pmol.

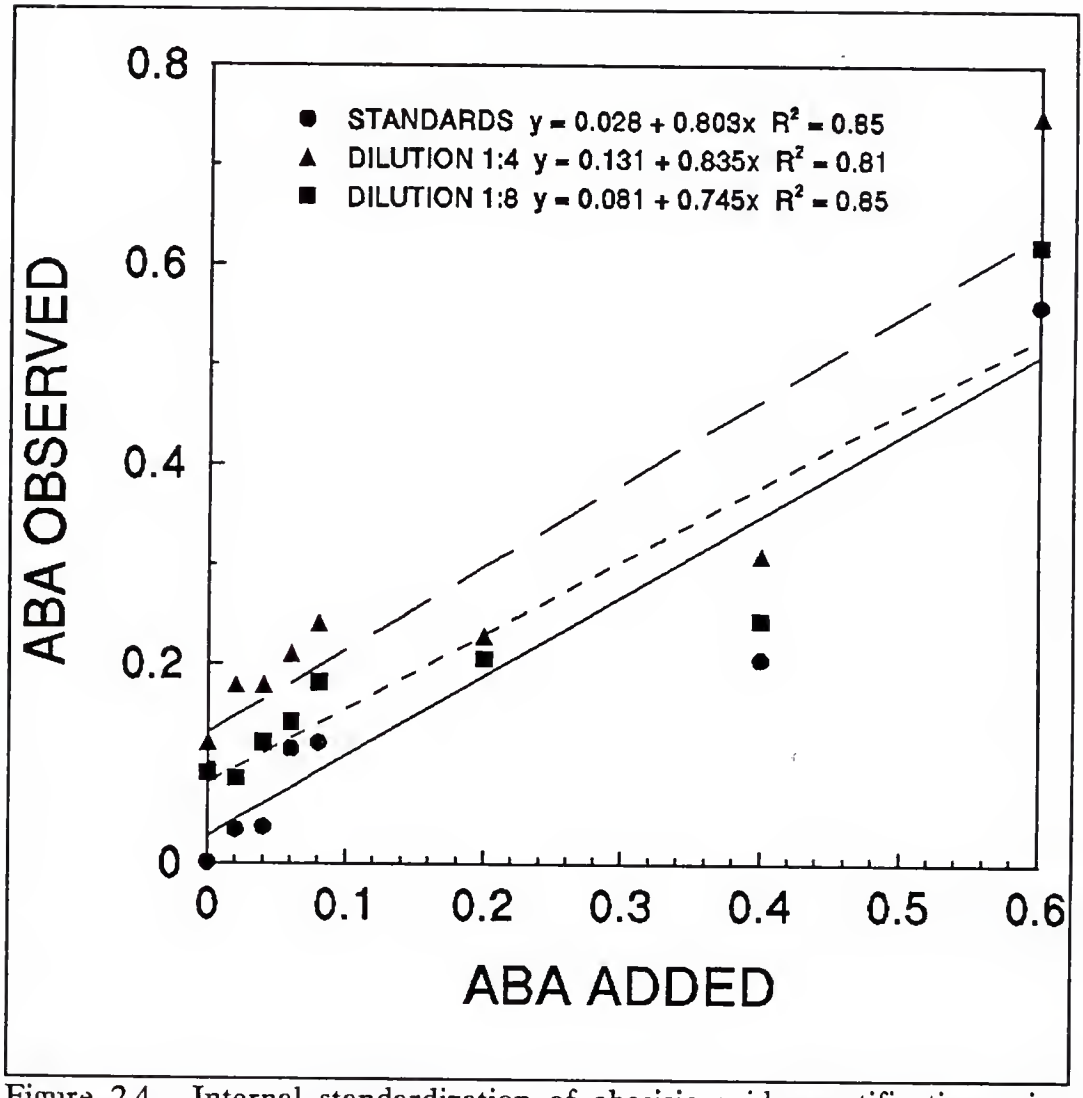

Figure 2.4. Internal standardization of abscisic acid quantification using ELISA with hydrilla turion tissue. Range of values was 0.02 to 0.6 pmol.

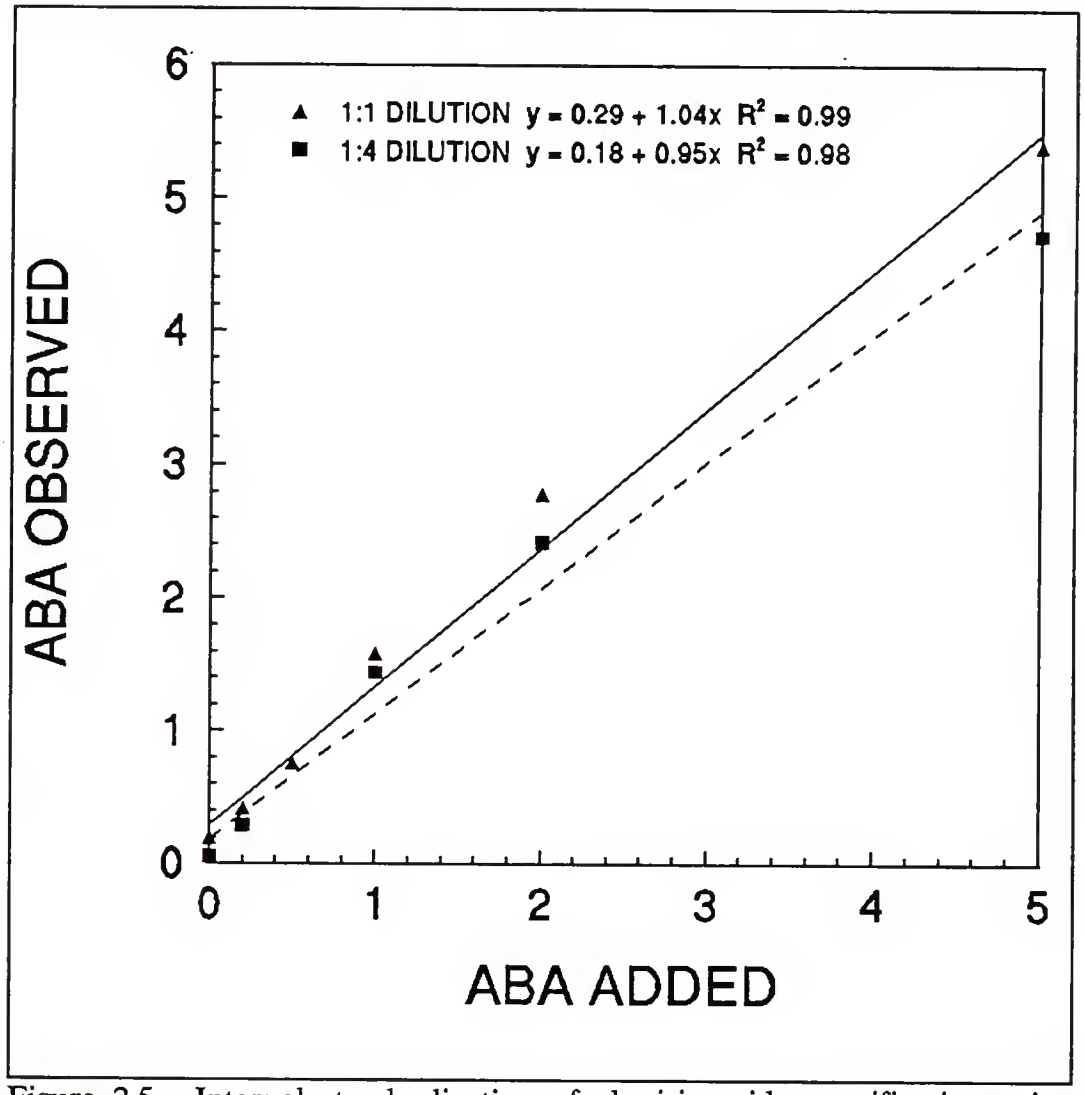

Figure 2.5. Internal standardization of abscisic acid quantification using ELISA with hydrilla turion tissue. Range of values was 0.2 to 5.0 pmol.

flexibility when undertaking ABA analysis from hydrilla tissue. Using this methodology, 8.0 pmol per sample for crown tissue (20 mg - dry weight) and 12 pmol per sample for turion tissue (8 mg - dry weight) were detected from these hydrilla tissue types. From these data, the appropriate amount of tissue needed for ABA extraction would be 10 to 20 mg - dry weight.

However, this procedure should be used to correlate large differences in ABA levels between tissue types, biotypes or seasonal hormone fluctuations. The minute quantities of ABA found endogenously can be depleted by a multitude of factors before actual analysis, therefore relative (large) differences only should be used when determining the effect of ABA.

CHAPTER III
THE INTERACTIVE EFFECT OF PHOTOPERIOD AND FLURIDONE ON THE GROWTH, REPRODUCTION, AND BIOCHEMISTRY OF HYDRILLA [*Hydrilla verticillata* (L.f.) Royle]

Introduction

Hydrilla is an exotic, submersed vascular plant that has become one of the most troublesome aquatic weeds in the state of Florida. Hydrilla was first observed in a Miami canal and in Crystal River around 1960 and is thought to have been introduced from Asia by the aquarium plant industry (Blackburn et al. 1969; Haller, 1976). Since introduction, hydrilla has spread throughout Florida, the southeastern United States, and California (Langeland, 1990). Infestations have also been reported in areas of Maryland, Virginia and Washington D.C., but this is thought to be a separate introduction involving a different hydrilla biotype (Stewart et al., 1984).

Hydrilla is a major problem in many freshwater ecosystems, forming a dense, entangled mat of shoots at the water surface (Haller and Sutton, 1975). This extensive growth interferes with most recreational water activities, navigation, and flood control. In addition, hydrilla effectively outcompetes most native vegetation, changing the natural balance of the aquatic ecosystem. Changes in the native flora and fauna occur that, ultimately impact the freshwater fishing industry of Florida.

Hydrilla regrows in the spring as the water temperature begins to rise (Haller, 1976). During the initial phases of growth, hydrilla shoots quickly reach the water

surface and begin to branch, forming a dense canopy (mat)(Van et al., 1977). This mat effectively absorbs nearly 95% of the available sunlight (PPFD), limiting light penetration to understory vegetation and reducing competition. In addition, hydrilla can adapt to extremely low light levels. Bowes et al. (1977) reported the light compensation point of hydrilla to be 12 to 20 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Further work by Salvucci and Bowes (1983) showed that a change in photosynthetic metabolism, from C_3 to C_4 - like photosynthesis occurred under certain conditions. This ability, coupled with the low light compensation point, allows hydrilla to outcompete other species and maintain the monotypic mat.

Hydrilla is difficult to control because spread can occur through a variety of mechanisms including stem fragmentation and turion development. In hydrilla, turions are produced at the tips of positively geotropic rhizomes, which extend from the root crown into the hydrosoil (Sculthorpe, 1967). These structures, termed subterranean turions or tubers, become detached from the mother plant and can remain dormant for 1 to 5 years (Van and Stewart, 1990). Turions may also be formed in leaf axils and are termed axillary turions (Yeo et al., 1984). Axillary turions are generally smaller than subterranean turions and are usually formed on detached, floating plants (Haller, 1976; Miller et al., 1993). Due to the smaller size, axillary turions are thought to function in dispersal, whereas subterranean turions act primarily in the persistence of hydrilla in a given area (Spencer et al., 1987; Thullen, 1990).

Hydrilla forms turions during the late summer and early fall in North Florida (Haller, 1976; Miller et al., 1993). Van et al. (1978) reported that hydrilla responded to photoperiod, with turions produced only under short-day conditions. Studies by Klaine and Ward (1984) elucidated the role of phytochrome in controlling this response. The level of ABA is thought to rise in response to short-day conditions, preceding turion formation. ABA applied exogenously to hydrilla under long-day conditions promotes turion production, but the differentiation between subterranean and axillary turion formation has not been studied (Van et al., 1978; Klaine and Ward, 1984).

Management of hydrilla is difficult and results are variable (Schmidtz et al., 1987), with control efforts focusing primarily on herbicide use for logistical reasons. Endothall, (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid), diquat, (6,7-dihydrodipyridol[1,2-α:2',1'-*c*]pyrazinediium ion), and copper sulfate alone or in combination (s) provide good initial control but hydrilla quickly regrows. A slow acting herbicide, fluridone, (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone), was introduced for aquatic weed control during the 1980's and has shown great promise for the control of hydrilla (McCowan et al., 1979). However, proper exposure periods and concentrations are necessary for effective control (Netherland et al., 1993).

Fluridone inhibits carotenoid biosynthesis, causing photooxidation of unprotected chlorophyll molecules (Bartels and Watson, 1978; Devlin et al., 1978; Maas and Dunlap, 1989). Fluridone has also been shown to inhibit the production

of ABA, which is thought to form from the carotenoid biosynthetic pathway (Li and Walton, 1990; Parry and Horgan, 1991). Because of this, fluridone is used in physiological experiments to study the effect of ABA on plant growth and development (Hole et al., 1989; Oishi and Bewley, 1990; Saab et al., 1990).

Short-term control of hydrilla is often realized within a given area but long-term eradication is rarely achieved. Fluridone provides excellent control of hydrilla and is generally applied in the spring as the hydrilla begins to regrow. Good initial control is observed but regrowth from dormant turions occurs during the mid to late summer months. This regrowth provides substantial biomass for reproduction in the fall and turion levels in the hydrosoil are replenished. Thus the cycle of spring treatment but regrowth and subsequent turion formation in the fall never results in effective, long-term management.

One management strategy would be to treat hydrilla during the fall before or during turion formation. However, hydrilla may not be sensitive to herbicides at this time. Herbicides are most active on young, rapidly growing plants. Hydrilla growing in the fall would be near maturity and should require a higher dose to achieve control. Fluridone has been shown to reduce turion formation at concentrations $\geq$ 5 ppb (MacDonald et al., 1993). However, the actual mechanism (i.e., reduction in ABA levels and subsequent turion inhibition or turion inhibition as a result of plant death), is not known. MacDonald et al. (1993) postulated that lower ABA levels result from sublethal fluridone concentrations and indicated a potential growth regulator phenomenon.

Clearly, the key in long-term hydrilla management is turion depletion in the hydrosoil. Two strategies have been suggested concerning this issue; inhibition of turion production or elimination of turion dormancy allowing precocious germination. The objective of this study focused on the former strategy. Hydrilla was grown under long (vegetative growth) and short-day (reproductive growth) conditions and evaluated for changes in growth, turion formation and biochemical parameters in response to varying rates of fluridone.

Materials and Methods

Hydrilla was initially planted on July 1, 1993 from apical stem segments and grown under natural conditions in 900 L concrete vaults at the Center for Aquatic Plants in Gainesville, FL. Stock hydrilla tissue was obtained from the Waccissa River in north Florida; a pre-determined "fluridone-free" water system. Treatment plants were grown in 10 x 10 cm plastic pots filled with organic potting soil[4] and covered with a 2 cm deep sand "cap" to prevent floating. Two 10 cm long apical hydrilla sprigs were planted per pot and 6 pots were placed in 24 x 30 cm plastic dishpans. On August 16, 1993 the plants were transferred to 540 L fiberglass vaults under short (natural) or long (artificially extended) day greenhouse conditions. Environmental conditions of the long-day greenhouse were 16 hr light/8 hr dark photoperiod, day temperature of 30 $\pm$ 5 C, night temperature of 25 $\pm$ 5 C, with a mean quantum

[4] Ace Hardware Corp., Oak Brook, IL 60521.

irradiance at noon of 1200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ (PPFD). Photoperiod was extended for 4 hours after sunset with incandescent bulbs [100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ (PPFD)]. The short-day greenhouse was maintained at similar conditions under natural (short-day) photoperiodic conditions.

A second population of young hydrilla plants was established on August 18, 1993 in a manner similar to the older plants and placed under the same conditions. The 8 week old population represented summer hydrilla growth while the younger plants represented hydrilla regrowing from overwintering turions. Throughout the remainder of this chapter, mature and young will be used to denote the 8 week old and newly established hydrilla plants, respectively.

Each greenhouse (photoperiodic condition) contained 8 vaults, 4 comprising mature hydrilla plants, 4 containing younger hydrilla. Each vault contained a total of 10 dishpans. Fluridone concentrations of 0, 1, 5, and 10 ppb were established within each population and photoperiodic regime beginning Sept. 2, 1993. Periodic flushing and insecticide treatment were necessary to maintain optimum growth conditions. After flushing fluridone was re-applied to each vault to achieve the desired concentration.

Hydrilla (one dishpan) was harvested from each vault on Sept. 2, 1993 and harvests continued on a weekly basis for 8 weeks. Each dishpan contained 6 replications (1 replication = 1 pot). Parameters measured from both plant age groups included fresh weight and subterranean turion number. Mature plants were subsequently divided into apical stem, crown, and subterranean turion sections.

Subsamples were a composite from all replications. Immediately following measurements and/or subsampling, young plants (entire plant) and mature plant subsamples were frozen in liquid nitrogen and stored at -20 C. Samples were lyophilized at -50°C and dry weights recorded. The tissue ground was then to 0.5 mm fineness and stored at - 20 C prior to biochemical analyses.

Chlorophyll/carotenoid analyses. Approximately 10 mg dry apical stem tissue was placed in a 20 ml glass scintillation vial and filled with 10 ml of chloroform:methanol (2:1 v/v). Vials were placed on a rotary shaker (150 rpm) for 12 hours at 4 C. The homogenate was filtered and the crude extract dried under a stream of nitrogen gas at room temperature under dim light ($< 5\ \mu mol \cdot m^{-2} \cdot sec^{-1}$). The residue was resuspended in 80% acetone and absorbances at 470, 646, and 663 nm were measured spectrophotometrically. Total chlorophyll concentration was calculated using the formula: $17.32 \times A_{646} - 7.18 \times A_{663}$. Total carotenoid content was determined using the formula: $1000 \times A_{470} - 3.27 \times Chl_a - 104 \times Chl_b)/229$ (Lichtenthaler and Wellburn, 1983). Chlorophyll *a* (Chl_a) and chlorophyll *b* (Chl_b) concentrations ($\mu g/mL$) were calculated from the following formulas: $12.21 \times A_{663} - 2.81 \times A_{646}$ and $20.13 \times A_{646} - 5.03 \times A_{663}$, for Chl *a* and *b*, respectively. Three replications of the composite apical stem section subsample from mature plants only (described above) were analyzed for chlorophyll and carotenoid content.

Anthocyanin analysis. Approximately 10 mg dry apical stem tissue was placed into a 20 ml scintillation vial filled with 5 ml of 1% acidic methanol (v/v). Tissue was extracted for 12 hours on a rotary shaker (150 rpm) at 4 C. The resulting

mixture was allowed to settle and absorbance of the supernate was measured spectrophotometrically at 530 and 657 nm. Chlorophyll has some absorbance at 530 nm in acidic methanol, therefore corrections were made using the formula: A_{530} - 0.25 x A_{657}, which assumes an extinction coefficient of 34,000 $M^{-1}cm^{-1}$ (Jonsson et al., 1984; Mancinelli, 1990). Anthocyanin content was determined from apical stem section subsamples from mature plants only (described above) with 3 replications per subsample.

Abscisic acid analysis. Tissue (10 to 20 mg dry weight) was extracted with 100% methanol for 12 hours on a rotary shaker (150 rpm) at 4 C. Methanol was evaporated at -50 C and the residue resuspended in 0.5 to 1.0 ml of tris-buffered saline and re-extracted as described previously. Following extraction, the homogenate was centrifuged at 9000 rpm for 4 minutes. Analysis for ABA was performed on the resulting supernate using an enzyme-linked immunoassay specific for ABA. Details of the immunoassay procedure are presented in Appendices A-E. A single sample from composite apical stem segments and crown subsamples of mature were analyzed for ABA content. A single sample from 3 replicates of younger plants 0, 2, 4, 6, and 7 weeks after treatment (WAT) were analyzed for ABA content.

Statistical analysis. Data were initially analyzed by analysis of variance to test for treatment effects (WAT, photoperiod, and fluridone concentration) and interactions. The effect of photoperiod was separated with Fisher's protected LSD test at the 0.05 level of significance. The effect of fluridone concentration was

compared to the untreated control using Dunnett's "T" test at the 0.05 level of significance. Means are presented with standard errors.

Results and Discussion

There was a significant ($P < 0.05$) three-way interaction between weeks after treatment, photoperiod and fluridone concentration on the dry weight of mature and young hydrilla plants. The dry weight of untreated plants increased over time in both photoperiodic regimes for both mature and young hydrilla populations (Tables 3.1 and 3.2). Young plants showed a greater increase in biomass than the mature hydrilla over the duration of the study. Seven WAT, the biomass of young plants increased by 83 and 90% under long and short-day conditions, respectively. However, mature plants only showed an increase of 39 and 25% for long and short day photoperiods, respectively. The dry weight of mature plants was reduced by fluridone concentrations of 5 and 10 ppb by more than 63%, regardless of photoperiod at 7 WAT. Young hydrilla plant biomass was reduced by fluridone concentrations of 5 and 10 ppb greater than 95% under short-day conditions and greater than 68% under long days. Fluridone at 1 ppb under long-day conditions caused 50 and 42% reduction in dry weight of mature plants compared to the untreated control after 6 and 7 weeks of treatment, respectively. Short-day grown mature hydrilla, however, did not show significant biomass reduction at 1 ppb fluridone, while younger plants were significantly reduced at 5 and 7 WAT under short day conditions.

Table 3.1. The effect of photoperiod and fluridone on the dry weight of mature hydrilla plants.

Photo-period[2]	Fluridone (ppb)	Weeks After Treatment[1]						
		1	2	3	4	5	6	7
		g/plant						
Long Day								
	0	4.6	3.9	11.9	9.6	3.4	7.4	7.5
	1	4.4	4.6	8.0	5.3	3.6	3.7*[3]	3.2*
	5	4.3	4.3	4.0*	3.5*	1.7	2.2*	1.1*
	10	4.2	3.9	5.6*	1.8*	4.1	2.8*	2.8*
Short Day								
	0	5.3	4.7	5.5	4.8	4.6	6.9	7.1
	1	4.3	4.1	5.1	3.5	4.1	6.0	4.3
	5	3.9	3.5	3.8	2.3*	1.7*	1.9*	1.4*
	10	4.9	3.3	4.5	1.6*	1.9*	2.3*	1.7*

[1] Average hydrilla plant dry weight at the initiation of the study was 4.25 g.

[2] $LSD_{0.05}$ = 2.3 to separate the effect of photoperiod within week and fluridone concentration.

[3] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

Table 3.2. The effect of photoperiod and fluridone on the dry weight of young hydrilla plants.

Photo-period[2]	Fluridone (ppb)	Weeks After Treatment[1]						
		1	2	3	4	5	6	7
		g/plant						
Long Day								
	0	0.17	0.17	0.40	0.36	0.58	0.75	0.66
	1	0.20	0.26	0.37	0.44	0.57	0.51*[3]	0.71
	5	0.20	0.25	0.21*	0.19*	0.10*	0.07*	0.03*
	10	0.30*	0.44*	0.20*	0.17*	0.23*	0.12*	0.21*
Short Day								
	0	0.28	0.43	0.55	0.60	0.90	1.38	2.97
	1	0.25	0.34	0.41	0.57	0.49*	0.86	0.81*
	5	0.33	0.43	0.42	0.42*	0.29*	0.27*	0.31*
	10	0.28	0.23*	0.18*	0.14*	0.07*	0.07*	0.05*

[1] Average hydrilla plant dry weight at the initiation of the study was 0.18 g.

[1] $LSD_{0.05}$ = 0.17 to separate the effect of photoperiod within week and fluridone concentration.

[2] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

There was a significant ($P < 0.05$) three-way interaction between weeks after treatment, photoperiod, and fluridone concentration on the subterranean turion production of mature and younger hydrilla plants. Turions were not produced during the first 4 weeks of treatment for either mature or young plants (data not shown). Mature hydrilla grown under short-day conditions produced a greater number of turions at 0 and 1 ppb fluridone 5 WAT or longer than under long-day conditions (Table 3.3). Subterranean turion production was reduced by more than 80% in mature hydrilla by fluridone at 5 and 10 ppb at 4 to 7 WAT under both photoperiods. Fluridone at 1 ppb also reduced turion production of mature plants at 4 to 7 WAT under long and short day conditions, with the exception of 5 and 7 WAT under short days.

Subterranean turion production by younger hydrilla plants was reduced under long day conditions and 5 and 10 ppb fluridone by nearly 100% (Table 3.4). Fluridone at 1 ppb reduced turion production in the younger plant 6 and 7 WAT under short and long photoperiods, respectively.

Young and mature hydrilla plants responded similarly to photoperiod and fluridone in terms of growth and reproduction. Fluridone is very effective for hydrilla control and rates of 5 and 10 ppb reduced dry weight and nearly eliminated turion production when hydrilla was exposed for greater than 5 weeks, similar to previous research (MacDonald et al., 1993). At 1 ppb fluridone, hydrilla growth was arrested in the mature plant population, while young plants at this treatment continued to accumulate biomass. Both mature and young plants gained biomass

Table 3.3. The effect of photoperiod and fluridone on the subterranean turion production of mature hydrilla plants.

Photo-period[1]	Fluridone (ppb)	Weeks After Treatment 4	5	6	7
		turions/plant			
Long Day					
	0	3.5	2.2	2.0	4.0
	1	0.3*[2]	0.3*	0*	0*
	5	0.2*	0*	0*	0.2*
	10	0.2*	0*	0.2*	0.5*
Short Day					
	0	4.3	6.3	8.0	11.7
	1	3.0	5.8	3.0*	9.0
	5	0.2*	0.2*	0.3*	2.3*
	10	0.2*	0*	0*	0.8*

[1] $LSD_{0.05}$ = 1.7 to separate the effect of photoperiod within week and fluridone concentration.

[2] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

Table 3.4. The effect of photoperiod and fluridone on the subterranean turion production of young hydrilla.

Photo-period[1]	Fluridone (ppb)	Weeks After Treatment			
		4	5	6	7
		turions/plant			
Long Day					
	0	0	0	1.7	0.7
	1	0	0.7	0*	0.7
	5	0	0	0*	0
	10	0	0	0*	0
Short Day					
	0	0.8	4.0	6.2	12.0
	1	3.3	4.2	5.0	4.0*
	5	0.2	0*	0*	0*
	10	0*	0*	0*	0*

[1] $LSD_{0.05}$ = 1.0 to separate the effect of photoperiod within week and fluridone concentration.

[2] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

in untreated conditions but the younger, more rapidly growing plants should have been more susceptible than mature plants to fluridone treatment.

A possible explanation for this apparent contradiction may be related to light intensity. Mature hydrilla plants have the majority of photosynthetic tissue near the water surface, exposed to a greater amount of light and potential photooxidative stress versus younger plant tissue emerging through the water column. In addition, the removal of apical dominance would cause branching at the water surface for the mature plant (Van et al., 1977). The new tissue produced by the mature hydrilla plants would then be more susceptible to the affect of fluridone due to the higher light intensity at the surface. This would result in a reduction of growth. However, the loss of apical dominance in young hydrilla resulting from fluridone treatment would cause branching near the hydrosoil, where light intensity and potential photooxidative stress is much lower. Hydrilla can survive under very low light intensities (Bowes et al., 1977) and this could explain the ability of the younger plants to increase in dry matter over the study period. Even though carotenoid levels would be lower as a result of fluridone treatment, so would potential photooxidative stress. Another explanation could be the rapid growth of the younger plants as compared to the mature hydrilla plants. The rapid growth could overwhelm the lethal affects of fluridone, with continued growth before fluridone began to take affect.

Subterranean turion production appeared to be directly related to growth in terms of dry matter for both young and mature plants, where reduction in growth also resulted in a reduction of turion number. There was turion formation under

long-day conditions by the mature hydrilla plants. However, the number of turions produced did not increase over time as in the short-day grown plants, which indicated a breach in long-day extension, or pre-induction before transfer to greenhouse conditions.

There was a significant ($P < 0.05$) three-way interaction between weeks after treatment, photoperiod and fluridone concentration for total chlorophyll, carotenoid and anthocyanin content. Chlorophyll and carotenoid content of the mature hydrilla plants was decreased at 5 and 10 ppb fluridone after 2 weeks of treatment in both long and short-day photoperiodic conditions (Tables 3.5 and 3.6). Chlorophyll and carotenoid content under long-day conditions was also decreased by 1 ppb fluridone, but the effect under short days was variable. There was also a general decline in these pigment levels as a function of weeks after treatment, with greater decreases observed at higher levels of fluridone. Conversely, anthocyanin content increased over time and short-day conditions (Table 3.7). The effect of fluridone on anthocyanin content was highly variable and anthocyanin content appeared to decrease anthocyanin content at higher fluridone rates.

Fluridone decreased carotenoid levels at concentrations of 5 and 10 ppb after 1 week of exposure as expected, as the mechanism of action of this compound has been demonstrated to be an inhibition of carotenoid biosynthesis (Bartels and Watson, 1978). Chlorophyll content also decreased in a similar fashion. Earlier work by Doong et al., 1993 demonstrated a 'lag' in the chlorophyll content of hydrilla at 5 ppb fluridone following carotenoid decrease but this was not evident from the

Table 3.5. The effect of photoperiod and fluridone on the chlorophyll content of mature hydrilla plants.

Photo-period[2]	Fluridone (ppb)	Weeks After Treatment[1]						
		1	2	3	4	5	6	7
		---------- μg/g-fresh weight ----------						
Long Day								
	0	673	847	679	695	543	450	489
	1	981*[3]	529*	322*	398*	170*	364*	---
	5	498*	310*	91*	94*	75*	198*	219*
	10	299*	167*	134*	25*	---	62*	207*
Short Day								
	0	616	613	527	465	438	269	486
	1	552	603	320*	489	278*	254	292*
	5	534*	357*	207*	225*	46*	52*	128*
	10	200*	85*	63*	278*	32*	29*	28*

[1] Average hydrilla chlorophyll content at the initiation of the study was 904 μg/g fresh wt.

[2] $LSD_{0.05}$ = 58 to separate the effect of photoperiod within week and fluridone concentration.

[3] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

Table 3.6. The effect of photoperiod and fluridone on the carotenoid content of mature hydrilla plants.

Photo-period[2]	Fluridone (ppb)	Weeks After Treatment[1]						
		1	2	3	4	5	6	7
		---------- μg/g-fresh weight ----------						
Long Day								
	0	68	74	86	92	87	65	92
	1	91*[3]	56*	44*	54*	24*	72	70
	5	49*	36*	15*	15*	12*	61	39*
	10	26*	21*	23*	3*	---	11*	42*
Short Day								
	0	67	67	74	84	77	48	106
	1	52*	63	44*	76	55*	46	47*
	5	54*	50*	25*	44*	9*	12*	26*
	10	19*	10*	10*	---	7*	9*	6*

[1] Average hydrilla carotenoid content at the initiation of the study was 89 μg/g fresh wt.

[2] $LSD_{0.05}$ = 10 to separate the effect of photoperiod within week and fluridone concentration.

[3] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

Table 3.7. The effect of photoperiod and fluridone on the anthocyanin content of mature hydrilla plants.

Photo-period[2]	Fluridone (ppb)	Weeks After Treatment[1]						
		1	2	3	4	5	6	7
		---------- μg/g-fresh weight ----------						
Long Day								
	0	58	76	69	76	123	76	113
	1	70*[3]	69	75	128*	68	66	156*
	5	52	41*	62	101*	94*	80	---
	10	54	53*	86*	141*	---	30*	---
Short Day	0	63	75	73	143	117	329	419
	1	56	55*	85	131	206*	346	300
	5	70	61*	143*	110*	95	81*	216
	10	51	50*	83	109*	94	99*	---

[1] Average hydrilla anthocyanin content at the initiation of the study was 63 μg/g fresh wt.

[2] $LSD_{0.05}$ = 39 to separate the effect of photoperiod within week and fluridone concentration.

[3] Values within a week and photoperiod followed by * are significantly different from the control (Dunnett's 't' test $_{0.05}$).

present study. A possible reason for this effect could be that high light intensity caused a greater degree of photooxidative stress and thus, more rapid chlorophyll breakdown. Chlorophyll content of untreated plants declined throughout the duration of the study in both photoperiodic regimes. Berg (1977) and Kar and Choudhuri (1987) reported a loss of chlorophyll content in hydrilla during late summer and hypothesized that a senescence had occurred. However, a decline in chlorophyll was also observed under long-day conditions, when senescence should not have been a factor.

The effect of fluridone on anthocyanin content was highly variable and did not cause an increase in the level of this pigment, as demonstrated by earlier studies (Doong et al., 1993). However, the level at which Doong et al., reported elevated anthocyanin content was 50 ppb fluridone, far above the levels tested in this study. Interestingly, anthocyanin levels increased dramatically under short-day conditions, with higher fluridone concentrations suppressing the level of this pigment. Berg (1977) reported an increase in anthocyanin content as hydrilla began to decline during late summer and this was probably the case for the short-day grown hydrilla plants. The decline in anthocyanin content from fluridone treatment probably resulted from tissue deterioration.

There was no significant differences in the ABA content of crown tissue from mature plants ($P > 0.05$ - see Appendix G). The average ABA content of these structures was 25 and 204 pmol/g - fresh weight for crown and turion tissue types, respectively. There was a significant interaction between weeks after treatment and

fluridone concentration for apical stem tissue but not a significant ($P > 0.05$) effect of photoperiod (Appendix H). Abscisic acid content increased in control and 1 ppb fluridone treated plants 1 and 2 weeks after study initiation, respectively, while fluridone at 5 and 10 ppb decreased ABA content (Table 3.8).

ABA content in younger plant tissue was only affected by photoperiod ($P < 0.05$), not by fluridone concentration and there was not an interaction (Appendix I). Abscisic acid content in young plant tissue was highest under short-day conditions when averaged over time and fluridone: 0.53 ± 0.07 and 0.71 ± 0.05 pmol/g-fresh weight, long and short days, respectively.

The results of the ABA analyses performed in this study are highly variable but follow similar findings of other researchers (Van et al., 1978; Klaine and Ward, 1984; Anderson et al., 1990 [unpublished]). ABA content was shown to rise soon after treatment, but the increase was transient. In addition, fluridone reduced ABA content and turion production in mature plants at 5 and 10 ppb, but this could also be the indirect consequence of plant death. ABA was higher under short-day length conditions, which favor turion formation. However, direct evidence linking turionformation and increased ABA content was not established. Although ABA is probably involved in some aspect of turion formation and/or development, the exact mechanism is still unknown.

The results from this study provided further evidence that hydrilla can be effectively managed by late summer to early fall applications of fluridone, which prevent turion formation. Mature hydrilla tissue was shown to be as susceptible to

Table 3.8. The effect of fluridone concentration on the abscisic acid content of mature hydrilla apical stem segments, averaged across photoperiodic regime.

Weeks After Treatment[1]	Fluridone (ppb) 0	1	5	10
	pmol/g-fresh weight[2]			
1	18	36	13	13
2	39	25	13	15
3	--	18	11	5
4	19	13	11	11
5	--	11	7	--
6	26	19	12	3
7	15	16	10	15

[1] Average abscisic acid content at the initiation of the study was 15 pmol/g fresh wt.

[2] $LSD_{0.05}$ = 13 to separate the effect of fluridone concentration within week.

fluridone as younger tissue and fluridone concentrations of 5 ppb or higher will dramatically reduce subterranean turion production. This contradicts the earlier hypothesis of MacDonald et al. (1993) who proposed that sublethal concentrations of fluridone could block turion formation. However, the present studies support the argument that lowering ABA concentration sufficiently to block reproduction would require a lethal reduction in carotenoids. Therefore, the reduction in turion formation is probably a coincidental result of plant death. Prudence should be exercised when applying these results to real-world situations, as factors such as exposure time and critical concentrations have yet to be evaluated.

CHAPTER IV
THE INFLUENCE OF FLURIDONE, PHOTOPERIOD AND PLANT GROWTH REGULATORS ON THE GROWTH AND REPRODUCTION OF MONOECIOUS AND DIOECIOUS HYDRILLA [*Hydrilla verticillata* (L.f.) Royle]

Introduction

Hydrilla is a submersed aquatic vascular plant that has become a major aquatic weed problem in the southeastern U.S. (Haller, 1976; Langeland, 1990). This species is particularly important in Florida, where it was introduced in the late 1950's by the aquarium plant industry (Blackburn et al., 1969). Since introduction hydrilla has spread throughout the southeastern United States and California.

There are both monoecious and dioecious biotypes of hydrilla which both can be found in the United States. Until recently, each biotype was believed to grow in distinct regions, but monoecious and dioecious have been reported in a common lake in North Carolina (Coley et al., 1993). Dioecious hydrilla exists primarily in those areas mentioned above, and consists of only the female plant. Monoecious hydrilla was introduced during the late 1970's and can now be found in areas of Virginia, Maryland, Washington D.C., and North Carolina (Stewart et al., 1984; Coley et al., 1993).

Hydrilla causes problems in many freshwater ecosystems, displacing most native vegetation. This highly aggressive species exists in large, monotypic stands, which form a dense mat at the water surface (Haller and Sutton, 1975; Bowes et al.,

1977). Hydrilla's extensive growth interferes with navigation and flood control, and severely limits most water recreation activities. Furthermore, hydrilla growth will alter the flora and fauna of a given area, impacting freshwater fish populations.

Hydrilla possesses several physiological and morphological adaptations including a low light compensation point, C_4-like photosynthetic metabolism, and specialized vegetative reproduction - [turions] (Bowes et al., 1977; Van et al., 1976; Salvucci and Bowes, 1983). Turions are specialized vegetative structures that function in persistence and dispersal of many aquatic macrophytes (Sculthorpe, 1967). In hydrilla, turions may be formed at the tips of rhizomes that penetrate into the hydrosoil (subterranean turions), or in leaf axils (axillary turions)[Yeo et al., 1984]. Subterranean turions are slightly larger and function to insure persistence of hydrilla within a given area, while the smaller axillary turions are thought to function in dispersal (Haller, 1976; Spencer et al., 1987; Thullen, 1990).

Hydrilla turion production varies between biotypes, from both a physiological and morphological standpoint. Dioecious hydrilla forms turions only under short-day (< 12 hours light) conditions, while monoecious hydrilla appears to be day-neutral (Klaine and Ward, 1984; Van et al., 1978; Anderson and Gee, 1986). Monoecious hydrilla will produce a greater number of smaller turions than dioecious hydrilla (Spencer and Anderson, 1987; Van, 1989). This size characteristic is true for both axillary and subterranean turions. Turion formation in the dioecious biotype is believed to be regulated by phytochrome (Klaine and Ward, 1984; Van et al., 1978). This response has been associated with increased levels of abscisic acid (ABA) and

exogenous applications of this hormone have resulted in turion formation under non-photoinductive conditions (Klaine and Ward, 1984; Van et al., 1978). However, delineation between axillary and subterranean turion formation was not addressed. Furthermore, the effect of ABA on monoecious hydrilla was not studied.

ABA has been associated with a number of plant processes including water relations, growth and development, and seed dormancy (Neill et al., 1986; Zeevaart, J.A.D. et al., 1988; Saab et al., 1990). ABA has also been shown to cause heterophylly in some aquatic plants (Kane and Albert, 1987; Goliber and Feldman, 1989).

Turion formation is similar to tuber formation in terrestrial species, such as potato (Solanum tuberosum) and jerusalem artichoke (Helianthus tuberosum). Tuber formation in potato involves changes in abscisic and gibberellic acid content, with an increase in ABA during tuberization and a subsequent decline in gibberellic acid levels (Krauss, 1985). Similar to turion formation in hydrilla, ABA will promote tuber formation under non-photoinductive conditions while gibberellic acid will inhibit tuberization under inductive conditions. However, most researchers feel there exists a critical ratio between these two hormones, not simply an 'on-off' phenomenon (Menzel, 1980; Vreugdenhill and Struik, 1989). This interactive effect has not been evaluated on either biotype of hydrilla.

Plant growth regulator experiments are usually performed under laboratory conditions to limit variability. Axenic culture is sometimes employed to study various plant processes under highly controlled conditions. Axenic culture has proven to be

a valuable technique for studying the physiology of aquatic plants (Kane and Albert, 1987). In addition, axenic culture permits plant growth under heterotrophic conditions. This allows for the potential use of a compound that influences photosynthesis and other autotrophic processes. Fluridone is often used to regulate ABA biosynthesis, but is lethal under autotrophic conditions.

Monoecious and dioecious hydrilla biotypes differ in the process of turion formation but little is known regarding the processes involved. Therefore, the objectives of these studies were to evaluate the growth and reproductive development of monoecious and dioecious hydrilla in axenic culture to 1) long and short-day photoperiod, 2) fluridone under long-day conditions, and 3) exogenous abscisic acid and gibberellic acid applications.

Materials and Methods

Axenic plant stock material was obtained from Dr. Michael E. Kane, Environmental Horticulture Dept., University of Florida. From this material, stock cultures of both biotypes were established from 3 to 5 cm long apical stem segments. Stock cultures were maintained in aluminum foil capped 0.94 L glass vessels filled with 150 cm^3 quartz silica sand (as a rooting medium) + 500 ml of liquid growth medium. Basal medium consisted of half-strength Murashige and Skoog mineral salts[1] and 0.75% (w/v) sucrose, adjusted to pH 6.5 with 1.0 N KOH prior to

[1] Murashige and Skoog Basal Salt Mixture (1962); Sigma Chemical Co., St. Louis, MO 63178.

autoclaving. Growth medium was autoclaved at 22 psi and 250 F for 20 minutes. Deionized water was added to the vessels containing sand prior to autoclaving to prevent breakage. After autoclaving, medium was added to the vessels and plant material transferred. All transfers were performed under sterile conditions. Stock cultures were maintained on a monthly basis and sterility was checked by indexing, according to the procedures of Kane (unpublished) and Knauss (1976). Stock cultures were maintained in an environmentally controlled growth chamber set at the following conditions: 11 + 1 hr light/6 + 6 hr dark photoperiod (1hr light interruption at the middle of the dark cycle to maintain long-day conditions); 150 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ (PPFD); 30 $\pm$ 5 C. The light source consisted of fluorescent and incandescent bulbs.

Experimental plant material was grown in 38 x 300 mm glass tubes[2] filled with 50 cm^3 quartz-silica sand and 175 ml growth medium. Plant material was obtained from stock plants. Establishment of hydrilla was accomplished by transferring a 3 to 5 cm long (0.005 g dry wt) apical stem section into each tube and forcing the basal end into the sand 1 to 2 cm deep to enhance rooting. Each tube was considered a replication. Experimental cultures were maintained in an environmentally controlled growth chamber set at the following conditions: 9 + 1 h light/7 + 7 h dark photoperiod (1 h light interruption at the middle of the dark cycle to maintain long-day conditions); 600 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ (PPFD); 30 $\pm$ 5 C mean light temperature and

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[2] Bellco Glass, Inc., Vineland, N.J. 08360.

25 ± 5 C mean dark temperature. Short-day grown plants were cultured in a chamber maintained at: 10 h light/14 h dark photoperiod -- 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ (PPFD) and 25 ± 5 C mean light temperature and 20 ± 5 C mean dark temperature. Lighting in both chambers consisted of a mixture of incandescent and fluorescent bulbs.

Photoperiodic response. Monoecious and dioecious hydrilla biotypes were grown under long or short-day conditions for 8 weeks with a minimum of 6 replications. The experiment was repeated once. After 8 weeks, the plants were harvested and subterranean and axillary turion number was determined. Subterranean turion development was determined by the formation a rhizome penetrating the sand substrate. Axillary turion formation was distinguished by a swelling of shoot tissue, compact leaf development and the lack of prominent internodes. The plants were then dried at 60 C for 48 h and dry weights recorded.

Effect of fluridone. A preliminary experiment was conducted to determine if hydrilla could be grown heterotrophically using axenic techniques with supplemental sucrose. Monoecious and dioecious hydrilla biotypes were grown under long-day conditions for 12 weeks. The experiment was repeated once with 4 replications. Fluridone was prepared as a concentrated stock in water from technical grade material[3] and filter-sterilized (0.22 μm)[4] prior to addition to the tubes. Fluridone was added at concentrations of 0, 0.5, 1.0, 5.0, or 10 ppb at the time of

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[3] DowElanco, Inc., Indianapolis, IN 46268.

[4] MSI, Fisher Scientific, Pittsburgh, PA. 15219.

establishment. After 12 weeks, the plants were harvested and the number of axillary turions produced was determined. The plants were then dried at 60 C for 48 h and dry weights recorded.

Effect of exogenous abscisic acid and gibberellic acid. Monoecious and dioecious hydrilla biotypes were grown under long-day conditions for a total of 8 weeks. Biotypes were run as separate experiments and each experiment conducted twice with a minimum of three replications. After 4 weeks of growth the growth medium was exchanged and hormone treatments were applied. Abscisic acid (99% mixed isomers)[5] and gibberellic acid (90% GA3)[5] were prepared as concentrated stocks in water and filtered-sterilized prior to treatment. Small amounts of ethanol was used to facilitate dilution and the appropriate ethanol concentration was added to the control plants. Abscisic acid was applied at 0, 0.1, 1.0, or 10 μM and gibberellic acid at 0, 5, 50, or 500 μM to establish a matrix of 16 total treatments. Four weeks after treatment, the plants were harvested and the number of axillary turions, subterranean turions, and flowers produced were counted. Axillary turions were subsampled and the plants dried at 60 C for 48 h and dry weights recorded. No attempt was made to distinguish between male and female flowers of the monoecious biotype.

Statistical analysis. Data were initially analyzed by analysis of variance to test for treatment effects (biotype, photoperiod, fluridone concentration, ABA or GA content) and interactions. Treatment means were separated with Fisher's

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[5] Sigma Chemical Co., St. Louis, MO 63178.

protected LSD test or Dunnett's 'T' test at the 0.05 level of significance. Means are presented with standard errors.

Results and Discussion

Although plant material was obtained from axenic stocks, a certain degree of contamination, bacteria and/or fungal, occurred in each of the experiments. Because of the relatively short duration of the experiments, this contamination did not cause plant death. There was no observed trend for any treatment to be more contaminated than the others. Therefore, it is not the author's intent to claim axenic culture was used or maintained, and conclusions drawn from these studies are not intended to reflect that premise.

There was a significant ($P < 0.05$) interaction between biotype and photoperiod for subterranean and axillary turion production and dry weight. Both biotypes produced greater biomass under long-day conditions (Table 4.1). Dioecious hydrilla grown under short days, produced less than 70% of the biomass produced by long-day grown plants. There was a 20% difference for monoecious hydrilla. Axillary turion production was not observed for dioecious hydrilla, while monoecious hydrilla produced these structures under both photoperiods (Table 4.2). Dioecious hydrilla produced subterranean turions only under short-day conditions, while the monoecious hydrilla produced subterranean turions under both photoperiods (Table 4.3).

In this study, photoperiod had a tremendous effect on plant growth and subterranean turion formation for dioecious hydrilla. Dioecious hydrilla has been

Table 4.1. The effect of photoperiod on the growth of 8 week old dioecious and monoecious hydrilla.

Photoperiod	Dioecious	Monoecious
	g dry/plant	
Long-Day	0.92±0.09[1]	1.21±0.05
Short-Day	0.28±0.04	0.95±0.05

[1] Means followed by standard errors.

Table 4.2. The effect of photoperiod on the axillary turion production of 8 week old dioecious and monoecious hydrilla.

Photoperiod	Dioecious	Monoecious
	turions/plant	
Long-Day	0	0.88±0.40[1]
Short-Day	0	2.71±1.41

[1] Means followed by standard errors.

Table 4.3. The effect of photoperiod on the subterranean turion production of 8 week old dioecious and monoecious hydrilla.

Photoperiod	Dioecious	Monoecious
	turions/plant	
Long-Day	0	0.75±0.49[1]
Short-Day	1.29±0.42	1.00±0.53

[1] Means followed by standard errors.

shown to undergo 'annual decline' (Berg, 1977), which is characterized by a loss of biomass prior to the onset of winter. In these experiments short days may have caused this phenomenon in dioecious hydrilla, thus limiting biomass production. Several species of plants cease vegetative growth during the onset of reproduction (Gardener et al., 1985). However, this does not seem to be the case with monoecious hydrilla. Another possible explanation could be the lower light intensity in the short-day chamber. Monoecious hydrilla also displayed significantly less biomass in this environment, but to a much lesser extent than dioecious hydrilla.

Dioecious hydrilla has been reported to be a short-day plant in terms of turion formation, with a critical photoperiod of 11 to 12 hours (Van et al., 1978; Miller et al., 1993). This characteristic response was also observed in these studies. However, axillary turion development was not observed under either day length. Haller (1976) and Miller et al. (1993) reported axillary turion formation occurred on floating hydrilla plants. Plants used in these experiments were intentionally rooted which possibly precluded axillary turion formation. Monoecious hydrilla produced axillary and subterranean turions under both photoperiods. This contrasts with earlier research, which showed no turion formation under 14 to 16 hour daylengths or photo-interruption of long nights (Anderson and Spencer, 1986; Spencer and Anderson, 1987).

Monoecious and dioecious hydrilla biotypes were adversely affected by increasing concentrations of fluridone (Table 4.4). Ten ppb fluridone caused 91 and 82 % reduction in shoot biomass of dioecious and monoecious hydrilla, respectively.

Table 4.4. The effect of fluridone on the growth of dioecious and monoecious hydrilla. Plants were grown under long-day conditions for 12 weeks.

Fluridone	Dioecious	Monoecious
(ppb)	---------- g dry/plant ----------	
0	0.56±0.18[1]	0.63±0.26
0.5	0.45±0.16	0.71±0.08
1.0	1.03±0.18	0.60±0.08
5.0	0.26±0.10	0.32±0.10
10	0.05±0.01*[2]	0.11±0.04*
$LSD_{0.05}$	0.38[3]	

[1] Means followed by standard errors.

[2] Values within a given biotype followed by * are significantly different from the control (Dunnett's 't' $test_{0.05}$).

[3] To separate the effect of photoperiod within a given level of fluridone.

One ppb fluridone caused a 46 % increase in dioecious shoot biomass. Fluridone decreased turion production at higher concentrations (5 and 10 ppb), while stimulating turion production at 0.5 ppb in monoecious hydrilla (Table 4.5). This observation is similar to results reported from earlier field experiments with dioecious hydrilla (MacDonald et al., 1993). This indicates that these biotypes behave similarly to fluridone in response to turion formation.

Fluridone is a very effective herbicide, inhibiting carotenoid biosynthesis and causing subsequent photooxidation of unprotected chlorophyll molecules (Bartels and Watson, 1978; Devlin et al., 1978). In this study, plant tissue became whitish in color, indicating pigment loss. Monoecious hydrilla appeared to 'recover' as indicated by green coloration, but still showed a decrease in biomass over the course of the study.

The lethal effect of fluridone has been demonstrated to be an inhibition of photosynthesis and plant death due to starvation. However, plants grown with sucrose amendments should have been able to survive fluridone treatment. If hydrilla could have been grown heterotrophically then fluridone could have been used to regulate ABA formation without the lethal effects from reduced photosynthesis. This could have allowed a determination of whether fluridone acts as a growth regulator at low concentrations or a herbicide at higher concentrations. Hydrilla could not be grown heterotrophically and therefore separation of the growth regulator and herbicidal effects of fluridone could not be ascertained.

In this experiment, fluridone decreased the growth of both monoecious and dioecious hydrilla biotypes, with monoecious being affected to a lesser degree than

Table 4.5. The effect of fluridone on the axillary turion production of monoecious hydrilla. Plants were grown under long-day conditions for 12 weeks.

Fluridone (ppb)	Turions/Plant
0	2.0±0.7[1]
0.5	5.0±1.1*[2]
1.0	2.7±1.7
5.0	0.3±0.3
10	0*

[1] Means followed by standard errors.

[2] Values followed by * are significantly different from the control (Dunnett's 't' $test_{0.05}$).

dioecious hydrilla. Although the goal of fluridone treatment in this experiment was to lower ABA levels thus precluding turion formation, it is possible that lowered ABA may be causing the reduction in growth. Studies with ABA-deficient mutants have demonstrated lower growth rates, epinasty and wiltiness associated with these types of mutations (Koornneef et al., 1982).

Fluridone at 5 and 10 ppb caused a deep purple coloration in dioecious hydrilla. Fluridone at high rates has been shown to increase the anthocyanin content of hydrilla but only in mature tissue (Doong et al., 1993). This phenomenon was thought to be in response to oxidative stress due to the loss of carotenoids. Anthocyanin production is also associated with high levels of carbohydrate (Creasy, 1968), present in mature tissue, and which was also present in this study.

There was not a significant ($P > 0.05$) effect of experiment for exogenous applications of abscisic and gibberellic acid, therefore experiments for each biotype were pooled. In dioecious hydrilla, exogenous applications of ABA alone induced axillary turion formation at all concentrations (Table 4.6). The greatest number of turions were observed following treatments of 0.1 and 1.0 μM ABA. The effect from ABA was reversed by applications of GA, but not completely. ABA also stimulated axillary turion production in monoecious hydrilla, but only at concentrations of 1.0 and 10 μM (Table 4.7). Similarly, GA at 50 and 500 μM completely reversed this effect at 1.0 μM ABA.

Total subterranean turion weight per plant reflected the same trend observed from turion number per plant (Tables 4.8 and 4.9). The size of dioecious turions were always larger than monoecious hydrilla regardless of treatment (0.005 and 0.002

Table 4.6. Axillary turion production by 8 week old, long-day grown dioecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	turions/plant			
0	0	0	0	0
0.1	15.6±5.1[2]	6.43±2.8	5.0±2.6	0
1.0	27.0±6.2	0.9±0.9	17.7±9.4	4.6±3.2
10	11.4±4.0	7.6±4.3	5.4±2.6	4.4±1.3

[1] $LSD_{0.05}$ = 10.6 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

Table 4.7. Axillary turion production by 8 week old, long-day grown monoecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	turions/plant			
0	0	0	0	0
0.1	0	0	0	0
1.0	42.6±14.7[2]	9.3±6.1	0	0
10	52.3±17.1	35.6±10.6	22.4±10.2	4.1±3.1

[1] $LSD_{0.05}$ = 4.8 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

Table 4.8. Axillary turion weight per plant by 8 week old, long-day grown dioecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	mg dry/plant			
0	0	0	0	0
0.1	74±31[2]	33±15	20±12	0
1.0	128±45	3±3	65±40	5±3
10	52±22	52±3	31±15	15±8

[1] $LSD_{0.05}$ = 0.06 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

Table 4.9. Axillary turion weight per plant by 8 week old, long-day grown monoecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	g dry/plant			
0	0	0	0	0
0.1	0	0	0	0
1.0	0.231±0.076[2]	0.020±0.013	0	0
10	0.146±0.052	0.057±0.025	0.042±0.03	0.006±0.005

[1] $LSD_{0.05}$ = 0.07 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

g^{-1} dry wt turion for dioecious and monoecious hydrilla, respectively). The percentage of total plant weight represented by turions also reflected the same response as described for turion number (Tables 4.10 and 4.11). Interestingly, monoecious hydrilla appears to partition a greater percentage of biomass for reproduction than dioecious hydrilla, producing a greater number of smaller turions.

The effect of ABA and GA on the shoot dry weight of both hydrilla biotypes was different than the effects on turion formation (Tables 4.12 and 4.13). Both ABA and gibberellic acid inhibited shoot growth of dioecious hydrilla with GA being the more inhibitory compound. At the highest concentration of both compounds, ABA ameliorated the activity of GA. Monoecious hydrilla was inhibited equally by ABA and GA in terms of shoot growth. However, these two compounds appeared to act additively to inhibit shoot growth. Although a reduction in shoot growth was observed on a dry weight basis, the plants treated with GA elongated to the top of the tube. It is probable that the reduction in dry weight was caused by decreased leaf production by the plants.

Dioecious hydrilla did not produce subterranean turions or flowers (data not shown). Monoecious hydrilla produced an average of 5 subterranean turions per tube only in the absence of hormone applications (untreated control). In addition, monoecious hydrilla produced an average of 44 flowers per tube the addition of gibberellic acid at 50 μM. Floral production has been reported to be influenced by nitrogen and phosphorus (Pietrse et al., 1984). Flowering has been associated with short day conditions in dioecious hydrilla, suggesting turion formation and flower

Table 4.10. Percent weight of axillary turions produced by 8 week old, long-day grown dioecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1]			
	0	5	50	500
	% of total dry weight			
0	0	0	0	0
0.1	12.0±6.2[2]	0.8±0.09	0.72±0.08	0
1.0	22.5±8.1	0.41±0.07	0.58±0.14	0.42±0.12
10	0.65±0.10	0.62±0.13	0.44±0.10	0.50±0.12

[1] $LSD_{0.05}$ = 8.95 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

Table 4.11. Percent weight of axillary turions produced by 8 week old, long-day grown monoecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	% of total dry weight			
0	0	0	0	0
0.1	0	0	0	0
1.0	31.2±9.3[2]	2.5±1.7	0	0
10	29.0±9.6	13.3±3.9	8.7±4.8	1.2±0.8

[1] $LSD_{0.05}$ = 9.8 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

Table 4.12. Shoot biomass production by 8 week old, long-day grown dioecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	g dry/plant			
0	0.80±0.04[2]	0.73±0.15	0.66±0.10	0.35±0.14
0.1	0.58±0.11	0.80±0.09	0.72±0.08	0.50±0.04
1.0	0.69±0.13	0.41±0.07	0.58±0.14	0.42±0.12
10	0.65±0.10	0.62±0.13	0.44±0.11	0.50±0.12

[1] $LSD_{0.05}$ = 0.08 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

Table 4.13. Shoot biomass production by 8 week old, long-day grown monoecious hydrilla as influenced by exogenous abscisic and gibberellic acid applications.

Abscisic Acid (μM)[1]	Gibberellic Acid (μM)[1] 0	5	50	500
	g dry/plant			
0	0.68±0.09[2]	0.53±0.12	0.72±0.07	0.49±0.07
0.1	0.42±0.03	0.58±0.07	0.54±0.10	0.34±0.06
1.0	0.58±0.11	0.47±0.12	0.42±0.11	0.31±0.05
10	0.46±0.05	0.37±0.05	0.33±0.06	0.32±0.06

[1] $LSD_{0.05}$ = 0.23 to separate the effect of abscisic acid within a given level of gibberellic acid or to separate the effect of gibberellic acid within a given level of abscisic acid.

[2] Means followed by standard errors.

production are linked to a common physiological process. However, floral stimulation by exogenous gibberellic acid, implied a different, separate mechanism was involved in this process.

Several researchers have demonstrated the effect of exogenous ABA applications on turion formation in dioecious hydrilla but little work has been done with the monoecious biotype (Klaine and Ward, 1984; Van et al., 1978). Dioecious hydrilla appeared to be more sensitive to ABA applications than monoecious hydrilla, as evidenced by turion formation at 0.1 μM and reduced sensitivity to GA applications. ABA has been shown to induce tuber formation in potato and this effect can be reversed by application of GA (Vreugdenhill and Struik, 1989). The increased sensitivity of the dioecious biotype to exogenously applied ABA could be related to photoperiodic responses, whereby applications of ABA could induce a cascade of reactions that lead to turion formation. However, this should have resulted in subterranean turion formation, not in the production of axillary turions.

Interestingly, monoecious hydrilla produced subterranean turions without treatment, similar to those plants in the photoperiod experiment. If ABA was directly responsible for the formation of these structures, then exogenous applications should have induced this process. This suggested an indirect role of ABA in turion development, with the formation of the rhizome being the initial step and not related to changes in ABA. ABA could be produced after rhizome differentiation to maintain dormancy and induce swelling of the rhizome tip. In a normally developing hydrilla plant, the amount of ABA produced during subterranean turion development

is sufficiently high that ABA could begin to accumulate in other tissues. These tissues could be an active meristem, such as the tips of stems or buds in the leaf axils. As ABA begins to accumulate, the tissue begins importing carbohydrate, forming a turion. ABA has been shown to regulate assimilate flow in plants (Porter, 1981) and could be acting to insure proper carbohydrate levels of the turion.

However, there appears to be critical ratio between ABA and GA, as evidenced by turion size. Turions produced under any influence of GA, were extremely small and probably would not have been able to regenerate a new plant. Therefore, ABA must accumulate in a limited number of sites, ensuring proper development of the propagule. This could be regulated by GA. Several researchers feel there exists a critical ratio of ABA to GA at all times within the plant, similar to the roles of auxin and ethylene (Menzel, 1980; Vreugdenhill and Struik, 1989). Therefore, the effect of exogenous ABA is probably not direct, but rather alters the ratio of ABA:GA. The relatively lower GA level induces the desired phenomenon (personal communication, Dr. Anwar Khan, Cornell University).

Collectively, these studies provided evidence that hydrilla cultured under controlled conditions responds similarly to hydrilla grown under field conditions. The exogenous applications of hormones indicated that ABA alone does not control hydrilla turion formation but rather a critical ABA:GA balance may be the key to the regulation of this phenological process. These hormones also appear to regulate floral production in monoecious hydrilla, and subterranean turion formation. However, the role of ABA and GA in these processes warrants further research.

CHAPTER V
SUMMARY AND CONCLUSIONS

These studies provided knowledge about the growth and turion formation process in hydrilla which is a submersed aquatic plant found throughout Florida, where it causes severe problems in infested areas. Insight into the turion formation process of this species could provide a basis for improving management programs.

The abscisic acid content of hydrilla tissue was determined by enzyme-linked immunoassay. Accurate quantification could be obtained from plant dilutions of 10 to 20 mg dry weight hydrilla tissue containing 0.02 to 5.0 pmol of abscisic acid. Although an effective solid-phase purification and concentration procedure was developed, no purification of the hydrilla extracts was necessary prior to quantification of abscisic acid with ELISA.

Short days promoted subterranean turion formation but this effect was reduced by long days and 5 and 10 ppb fluridone. Fluridone caused a significant reduction in chlorophyll and carotenoid levels but the effect on anthocyanin content was variable. Short days caused elevated anthocyanins and this effect was diminished by fluridone. Fluridone reduced the abscisic acid content of mature plant apical stems and abscisic acid content was greater under short days in younger plants. These studies provided further evidence that fluridone can be used in a fall herbicide treatment to reduce turion production but, the ability of fluridone to inhibit turion

production appears to be a coincidental result of plant death.

The growth of monoecious and dioecious hydrilla was reduced under short-day conditions. Monoecious hydrilla formed turions under long and short day conditions, while dioecious hydrilla formed turions only under short-day conditions. Fluridone reduced the growth of both biotypes and reduced turion formation in monoecious hydrilla. Exogenously applied abscisic acid at 0.1 μM or higher induced turion formation of dioecious hydrilla under long day conditions, but this could be partially reversed by exogenously applied gibberellic acid. Abscisic acid also induced turion formation in monoecious hydrilla, but concentrations $\geq$ 1.0 μM were necessary to achieve results similar to those for dioecious hydrilla. This study indicated abscisic acid alone does not control hydrilla turion formation, but rather a balance between abscisic acid and gibberellic acid is the key to regulation of this phenological process.

APPENDIX A
ELISA PLATE COATING PROCEDURE

1. Dissolve 10 mg of rabbit anti-mouse IgG antibody[1a] in 40.5 mls of sodium bicarbonate[A] buffer and wait 5 minutes. Dispense 200 μL into each well of two - 96 well microtiter plates[2a].

2. Cover with plastic wrap or aluminum foil and incubate at 4 C for 24 hours.

3. After 24 hours, decant the solution in each well and wash each well with 200 μL of wash solution[B]. Repeat this step for a total of 3 washes, decanting each wash at a time. After the last wash, dry by inverting on an absorbent paper toweling or large Kimwipes[2b].

4. Dissolve 2 mg of monoclonal ABA antibody[3] into 40.5 mls of sodium bicarbonate buffer[A] and wait 3-5 minutes. Dispense 200 μL in each well of the above plates. Incubate for 36 to 48 hours at 4 C.

5. After incubation, decant the solution and repeat the wash and drying procedure in step 3.

6. Dissolve 400 mg of rabbit serum albumin[1b] in 41 mls of TBS buffer[C] and add 200 μL to each well. Cover (keep dark) and incubate for 1 hour at room temperature.

7. Decant the unbound albumin and wash the plate again, repeating the wash procedure in step 3. Dry the plate and it is ready for use. **NOTE: The plate must be used within 30 minutes of this step.**

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[1] Sigma Chemical, St. Louis, MO 63178.
[a] Catalog # M-9637 [b] Catalog # A-0639

[2] Fisher Scientific, Pittsburgh, PA 15219.
[a] Catalog # 08-757-23 [b] Catalog # 06-666-1B

[3] Phytodetek™, Idetek, Inc., 1245 Reamwood Ave., Sunnyvale, CA 94089. Catalog No. 8015.

APPENDIX B

ELISA SAMPLE QUANTIFICATION

1. Mix tracer solution by reconstituting 4 vials of tracer[4], each with 1 ml of D.I. water. Mix gently and wait 5 minutes. Transfer the contents of the vials to 17 mls of tracer buffer[E] and mix. Be sure to rinse the tracer vials with the buffer solution to remove all tracer.

2. Add 100 μL of sample or standard to the wells on the plate.

3. Next add 100 μL of tracer solution to each well and mix gently by tapping the sides of the plate. Cover and incubate for 3 hours at 4 C.

4. Prior to the end of the incubation period (5 - 10 minutes), dissolve 8 pNPP[5] tablets in 41 mls of substrate buffer[D] (1 tablet/5 mls buffer).

5. After incubation, decant the sample or standard and repeat the wash procedure in step 3. Dry the plate and add 200 μL of substrate buffer solution to each well. Incubate the plate for 45 to 60 minutes at 37 C.

6. After 45 minutes, check the absorbance of the zero ABA standard[6] at 405 nm. The absorbance for the zero (TBS alone) should be just over 1.0 (actually from 1.0 to 1.2). If the absorbance is lower, allow the plate to incubate for a while longer.

7. After the above criteria is met, add 50 μL of 1 N KOH to each well to stop the reaction. **DO NOT DECANT THIS SOLUTION.** Wait 5 minutes, measure and record the absorbances.

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4 Phytodetek, Idetek, Inc., 1245 Reamwood Ave., Sunnyvale, CA 94089 Catalog No. 8019.

5 Sigma Chemical, St. Louis, MO 63178. Catalog # 104-105.

6 Hyperion Micro-Reader, Model 4025. Hyperion, Miami, FL. 33186.

APPENDIX C

ELISA STOCK SOLUTIONS AND STANDARDS

1. Dissolve 0.0264 g of abscisic acid into 100 ml of HPLC grade methanol to make a 1 mM ABA solution.

2. Add 100 μL of 1mM ABA to 100 ml methanol to make a 1 μM ABA solution.

3. Add 100 μL of 1 μM ABA to 100 ml methanol to make a 1 nM ABA solution.

4. Quantification is based on pmol per 0.1 ml, therefore, a 1 nM ABA solution contains 100 pM of ABA per 0.1 ml. Base further dilutions on this premise and make several standards (0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10 pM/0.1 ml). Save all dilutions.

5. Add 1 ml of standard prepared in methanol (step 4) into a scintillation vial and evaporate the solvent. Reconstitute with 1 ml of TBS buffer and vortex for 30 seconds - making the standard for the plate.

NOTE: Make all dilutions using volumetric flasks, bring all solutions up to volume.

APPENDIX D

ELISA BUFFERS AND SOLUTIONS

NOTE: Use only distilled, deionized water when making solutions.

A. Sodium bicarbonate (50 mM), pH 9.6.

B. Wash Buffer, pH 7.0
- 0.85% NaCl
- 0.05% TWEEN 20
- 0.1% Sodium Azide

C. Tris-Buffered-Saline (TBS buffer)
- 100 mM Tris-HCl stock (pH 8.5)
- 100 mM NaCl
- 1 mM $MgCl_2$
- 0.1% Sodium Azide

Adjust to pH 7.5.

D. Substrate Buffer (pH 9.6)
- 9.6% diethanolamine (98%)
- 0.5 mM $MgCl_2$

E. Tracer Buffer - TBS buffer (step C) + 0.1 % gelatin.

APPENDIX E

ELISA STANDARD CURVE AND SAMPLE ABA DETERMINATION

1. Take the average of the zero ABA (Bo term) and the 100 pM (NSB) replications. The Bo should range from 1.0 to 1.3 while the NSB will be from 0.07 to 0.115 in absorbance values.

2. Use the following formula: ((standard - NSB) / (Bo - NSB)) * 100, to determine % binding for the standards.

3. Calculate the **logit** of % binding term using the following formula: LN (%binding/(100 - %binding)).

4. Using the logit term generated above as the independent variable and the natural log of the ABA standard (concentration added) as the dependent variable, generate a regression equation for the standards.

5. Substitute the logit term for x and solve, generating the natural log of the predicted ABA concentration. Take the anti-nlog of the above value (e^x) to produce the predicted concentration.

7. To obtain sample ABA content, substitute the sample absorbance for the standard absorbance in step 2. Perform step 3 and skip to step 5, entering the logit value into the standard curve regression equation and take the anti-nlog to obtain sample ABA content.

8. The above value generated is the amount of ABA per the 100 μL, therefore divide by 0.1 and multiply by the dilution factor used to obtain sample ABA content.

APPENDIX F

ABSCISIC ACID COLUMN EXTRACTION PROCEDURE

1. extract sample with 10 mls of 100% methanol in glass homogenizer, filter under suction.
2. dilute filtered extract to 20% MeOH with acidic water (1% formic acid).
3. pass through C_{18} column, ABA and polar compounds will be retained on the column, water soluble compounds will elute off, discard eluant.
4. elute column with 15 mls of 40% MeOH (unacidified), retain eluant; ABA will elute off but most polar compounds will remain on the column.
5. dilute the above eluant to 10-20% MeOH with acidic water (50-60 mls) and pass through another C_{18} column. ABA will again be retained on the column.
6. elute with 10 mls of 100% MeOH or 100% acetone, collect eluant for quantification.
7. if large quantities of samples are to be extracted, the second column used in steps 5 & 6 can also be used as the initial column in step 3.

APPENDIX G

THE EFFECT OF PHOTOPERIOD AND FLURIDONE ON THE ABSCISIC ACID CONTENT OF MATURE HYDRILLA ROOT CROWNS.

Photo-period	Fluridone (ppb)	Weeks After Treatment 1	2	3	4	5	6	7
		---------- pmol g^{-1} fresh weight ----------						
Long Day	0	16	95	13	29	33	20	19
	1	15	29	21	17	16	25	19
	5	24	16	23	--	18	30	10
	10	17	28	29	17	19	22	14
Short Day	0	24	27	13	30	44	23	16
	1	123	46	42	21	22	25	--
	5	36	14	20	24	25	19	11
	10	2	42	27	10	23	12	9

APPENDIX H

THE EFFECT OF PHOTOPERIOD AND FLURIDONE ON THE ABSCISIC ACID CONTENT OF MATURE HYDRILLA APICAL STEM SEGMENTS.

Photo-period	Fluridone (ppb)	Weeks After Treatment 1	2	3	4	5	6	7
		---------- pmol g^{-1} fresh weight ----------						
Long Day	0	11	47	11	12	1	14	10
	1	42	28	13	8	8	16	12
	5	14	11	9	8	7	--	12
	10	14	19	1	11	--	3	--
Short Day	0	25	31	4	26	5	38	20
	1	30	23	22	18	14	21	21
	5	12	15	12	15	--	12	10
	10	11	11	9	--	--	4	15

APPENDIX I

THE EFFECT OF PHOTOPERIOD AND FLURIDONE ON THE ABSCISIC ACID CONTENT OF YOUNG HYDRILLA.

Photo-period	Fluridone (ppb)	Weeks After Treatment			
		2	4	6	7
		---------- pmol g^{-1} fresh weight ----------			
Long Day	0	0.30	0.62	---	0.76
	1	0.16	0.32	0.96	0.58
	5	0.87	0.27	---	---
	10	0.80	0.44	0.82	0.78
Short Day	0	1.15	0.52	1.70	0.62
	1	0.77	0.26	1.28	0.29
	5	0.67	0.41	0.65	1.14
	10	1.02	0.41	---	---

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BIOGRAPHICAL SKETCH

Gregory E. MacDonald was born on October 14, 1963, in Geneva, New York. During his youth he spent many hours working his relatives' farms, milking cows and repairing farm machinery. He graduated from Geneva High School in 1981 and received an Associate of Applied Science in agricultural engineering from Alfred State University. In May of 1986, he received a Bachelor of Science from Cornell University in vegetable crop production.

In April of 1987, he enrolled at the University of Florida and was awarded a Master of Science in agronomy in 1991. He continued his career at the University of Florida in 1991, to pursue a Doctor of Philosophy under the supervision of Dr. Donn Shilling. He is active in the Southern Weed Science Society, Weed Science Society of America, Florida Weed Science Society, and Aquatic Plant Management Society.

On October 30, 1993, he married Miss Michelina Carter and the couple plan to move to Tifton, Georgia, upon completion of his degree. His hobbies include bowling, camping, fishing, and antique agricultural machinery restoration.

I certify that I have read this study and that in my opinion it conforms to acceptable standard of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standard of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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